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PRINCIPAL INVESTIGATOR: Dr. David Eick

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Dr. David Eick (corresponding PI: Dr. Lynda Bonewald)

E-Mail: eickj@umkc.edu

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Even though commercial bone cements have not significantly changed in the past 50 years and have been used throughout the world, there are significant drawbacks with the current systems. We have developed a silorane based resin superior to polymethyl methacrylate (PMMA) with many improved properties such as significantly less polymerization stress without an associated reduction in mechanical properties. The specific aims for this project are: Specific Aim 1: Develop a silorane bone cement suitable for in vivo studies and to optimize the formulation of the chemically and mixed cured cement prototypes. Specific Aim 2: Determine the biocompatibility properties and wear debris generation of silorane bone cement prototype. Specific Aim 3: Determine the biological response to silorane bone cement prototype in animal models. By addressing the shortcomings of current PMMA bone cement, the development of the novel silorane bone cement will result in a paradigm shift in orthopedic biomaterials.

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Yearly Report

Project Title: Bone Repair and Military Readiness

INTRODUCTION:

Even though commercial bone cements have not significantly changed in the past 50 years and have been used throughout the world, there are significant drawbacks with the current systems. These include toxicity, contraction with polymerization, and heat generation. We have developed a silorane based resin superior to polymethyl methacrylate (PMMA) with many improved properties such as significantly less polymerization stress without an associated reduction in mechanical properties. These new resins do not generate cytotoxicity, antigenicity, polymerization stress or significant heat generation. In addition, it appears that this new bone cement is actually supportive of new bone formation. Orthopedic surgeons have had to adapt surgical techniques to account for issues with cementing total joint prostheses and subsequent total joint failures. The cement-bone interface is problematic, as there is no true bonding of cement to bone, only interlay in the trabecular spaces. A cement that can achieve true integration with the bone surface would be advantageous in that it would improve stress transfer to bone and decrease particulate wear. This integration, in turn, could result in improved bone stock if the need for revision arises. Bone infection with prothetic devices is an increasing major medical problem. As the proposed bone cement prototype polymerizes at a much lower temperature, antibiotics that are sensitive to heat can be added to the cement. Currently, only tobramycin, gentamycin and vancomycin are heat-stable and survive the heat generated by commercially available bone cement during polymerization. Therefore, a wider spectrum of antibiotic availability in bone cement may allow for more appropriate treatment of patients. By addressing the shortcomings of current PMMA bone cement, the development of the novel silorane bone cement will result in a paradigm shift in orthopedic biomaterials.

BODY:

The specific aims for this project are:

Specific Aim 1: Develop a silorane bone cement suitable for *in vivo* studies and to optimize the formulation of the chemically and mixed cured cement prototypes.

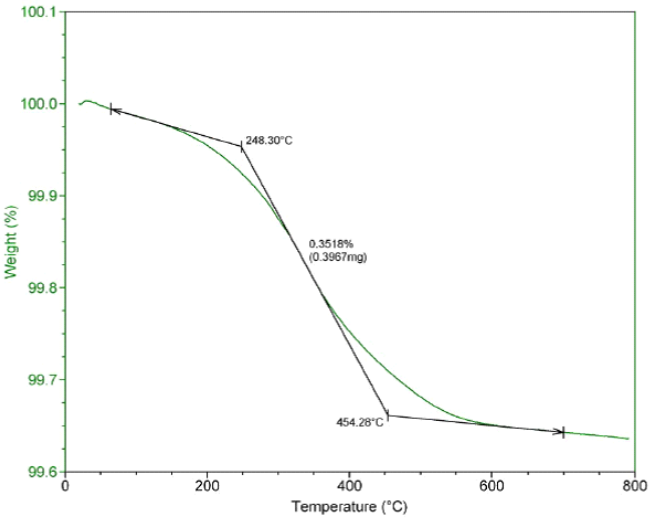
Specific Aim 2: Determine the biocompatibility properties and wear debris generation of silorane bone cement prototype.

Specific Aim 3: Determine the biological response to silorane bone cement prototype in animal models.

Progress one year to date:

FY10 Task 1 Develop a silorane bone cement suitable for in vivo studies and to optimize the formulation of the chemically and mixed cured cement prototypes, <u>Subtask 1a. Silanization of filler particles.</u> Months 1-12.

A). Generation of 1TOSU DY5. 1TOSU modified DY5 macroparticle glass (299g) was transferred to UMKC on 8 May 2012. The original powder has d50 of 3.266 μm and measured specific surface area of 51100 cm^2/mL (for DY5: $\rho = 2.55g/mL > 2.003m^2/g$). Modification resulted in a weight loss of 0.3518% by mass due to volatile organic carbon material or a surface area per TOSU group of 21.6 $Å^2$/group to 43.6 $Å^2$/group. This result is dependent on the percent of moisture that is attributed to the modified sample since no distinct moisture loss transition is observed. See figure 1.



TGA calculation:

$$\frac{\text{surface area}}{\text{group}}\left(\frac{\text{Å}^2}{\text{group}}\right) = \frac{2.003\ \text{m}^2/\text{g} \times \left[\%\text{N.V.} - \frac{\%\text{volatile}}{M_v}(M_{NV})\right] \times \left(\frac{1\times10^{10}\ \text{Å}}{\text{m}}\right)^2}{\frac{\%\text{volatile}}{M_V} \times 6.022\times10^{23}}$$

Figure 1. TGA calculation: where %N.V. is % nonvolatile (=mass nonvolatile), % volatile (= mass volatile), 2.003 m^2/g = surface area per gram glass, Mv= molecular mass of volatile portion = 229.3 g/mol, and MNV = molecular mass of non-volatile portion of 1-TOSU = 60.1 g/mol.

B). Generation of 3TOSU DY5, 1TOSU M12, and 3TOSU M12. Filler particles were reacted with silanes, washed to remove excess reagent, cured in an oven, packaged, analyzed and delivered to UMKC for composite manufacture and testing. The 3TOSU modified DY5 macroparticle glass (299g) and 1TOSU and 3TOSU modified M12 glass powder (149 g each) was transferred to UMKC on 25 July 2012. The original DY5 powder has d50 of 3.266 μm and measured specific surface area of 51100 cm^2/mL (for DY5: ρ = 2.55g/mL and SSA = 2.003m^2/g) while the M12 glass powder has a d50 of 2.85 μm and a measured surface area of 19250 cm^2/mL (for M12 powder: ρ = 2.83g/mL and SSA = 1.93m^2/g). Chemical modification with TOSU organic groups resulted in weight losses of 1.20, 1.47, and 1.77 mg per gram of glass by mass due to volatile organic carbon material. From these data, surface areas per TOSU group of 71.3, 49.7, and 46.4 $Å^2$/group were calculated. This result is independent on the percent of moisture attributed to the unmodified or modified glass powder samples since data were normalized to dehydrated sample weight losses. See figure 1 below, which shows TGA data for samples submitted to UMKC for composite testing. Expected values for organic group density of 50 $Å^2$/group were readily obtained with the one exception of 3TOSU DY5 glass powder; however, the group density reproduces that obtained in previous work for the 3TOSU reaction with DY5 powder.

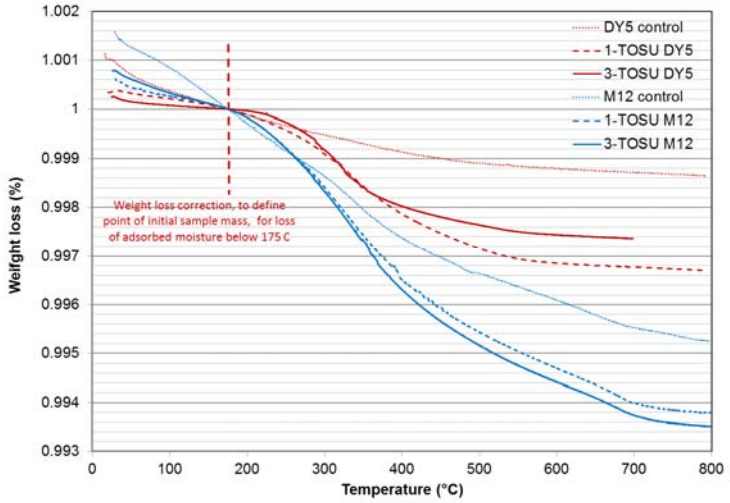


Figure 2: Thermogravimetric analysis of surface-modified fillers, where heat-induced weight loss of the surface-bound organic group is quantified. The mass of organic groups is used to calculate moles of groups per surface area resulting in a determination of the area per group occupying the filler surface and a quality of surface modification.

glass baseline loss	#TOSUglass	% nonHOH sample	% nonHOH wt loss	glass surface area (m^2/g)	glass density (g/cc)	mole mass whole group (g/mol)	mole mass volatile group (g/mol)	mole mass non-volatile group (g/mol)	area per group (A^2/group)
0.0014	1 DY5	1	0.00175	2.003	2.55	281.38	229.3	52.08	43.6
0.0014	3 DY5	1	0.0012	2.003	2.55	309.43	257.4	60.1	71.3
0.00473	1 M12	1	0.00147	1.925	2.83	281.38	229.3	60.1	49.7
0.00473	3 M12	1	0.00177	1.925	2.83	309.43	257.4	60.1	46.4

Table 1. Thermogravimetric data and calculations table showing surface group densities for DY5 and M12 glass powder samples. Group densities of ~50Å^2 per group are considered a dense surface coverage.

FY10 Task 1: Develop a silorane bone cement suitable for in vivo studies and to optimize the formulation of the chemically and mixed cured cement prototypes, Subtask 1b. Optimize composite formulation with respect to mechanical/handling properties. Months 13-24.

A). Mixing and Storage to Improve Stability of Formulation. Progress has been made with respect to the mixing technique using M12 glass filler. We have found that the current silorane bone cement formulation is stable when divided into two components: one containing silorane resin and Lamoreaux's catalyst, the other containing silorane resin, fillers, and the light initiation system (PIH, EDMAB, and CQ). The two components have been shown stable up to a week and when mixed together the material polymerizes within an hour. Further modification of the amounts in each component should decrease polymerization time and improve overall handling properties.

Progress has also been made with respect to improving the reproducibility of the mixing technique and storage of the chemical catalyst LMC. We have identified potential sources of variability in the mixing process and are systematically determining acceptable limits to improve our mixing protocols. For example, the time required to add LMC to the bone cement prior to polymerization was studied. Preliminary results suggest that a longer addition time may decrease modulus of elasticity in addition to increasing overall variability, however further testing is required to confirm these findings.

Progress has been made with respect to improving the reproducibility of the mixing technique and storage of the chemical catalyst LMC. We have investigated the stability of LMC in hopes of identify optimal storage conditions. We identified an optimal storage condition for the LMC, which ensures stability throughout a lot of material. For each new preparation of catalyst, it is transferred into individual use vials (~1 – 3 mL), placed under inert atmosphere, and stored in the freezer (< 5 °C). The vials are warmed to room temperature in a desiccator before use. This method has been found to be effective for ensuring the first and last sample of a batch are "identical". To ensure that this is the case, a protocol was developed to test the formulation before using the material in rat pull out tests. This test has been used to monitor the efficacy of the formulation (e.g., reactivity of Pt-catalyst, reactivity of new filler lots and modifications).

B). ***Quality Control – Quality Assurance***

There is a need for standardizing the protocols and sample preparation so that all synthesis, formulations, and testing are performed the same way with the same amounts for each component. Also, storage of all components is being investigated.

1. **Photoinitiation system:** With the exception of LMC, the initiation components are all solids. It is a reactive Pt-based catalyst. Other than storage in the freezer, no shelf-life dates with no other precautions are taken.
3. **Handling of LMC:** For each new preparation of catalyst, it is transferred into individual use vials (~1 – 3 mL), placed under inert atmosphere, and stored in the freezer (< 5 °C). The vials are warmed to room temperature in a desiccator before use. .
4. **Shelf life of LMC:** To test the stability of the LMC in ambient conditions, five vials were prepared as previously described and placed on the bench top in the lab. They will be tested at one-month intervals with freezer samples for reference.
5. **Testing:** A protocol was developed to test the formulation before using the material in rat pull out tests that we refer to as the 'mimic pull-out test'. This test will be used to monitor the efficacy of the formulation (e.g., reactivity of Pt-catalyst and effect of humidity on the modified and unmodified fillers).
6. **Initial Testing:** We will compare the reactivity of two different batches of catalyst (new – one week and seven month synthesis date).

C). Mechanical Testing using the *Ex Vivo* Pull-Out test and a newly developed Pull-Out Bone Mimic Test for Prototype Bone Cements.

Experiment 1. This rat femoral ex vivo test was performed as described: Frozen, male Sprague–Dawley rats (approximately 6 months old) were used (n=10 rats, 20 femora). A hole was drilled into the intercondylar notch with a Dremel drill bit to penetrate the subchondral cortical bone and gain access to the femoral intramedullary canal. The marrow cavity was disrupted by inserting a threaded hand drill proximally through the entire length of the diaphysis to approximately the level of the lesser trochanter. A guide implant was placed into the ablated cavity to ensure that the canal was an appropriate size to accommodate the definitive implant. Three silorane bone cement formulations, containing either ECHE M12, 1TOSU M12, or 3TOSU M12 surface treated glass fillers, were compared with commercial bone cement (positive control). The cements were introduced into the intramedullary canal and then a titanium implant, 22 mm long and 1.5 mm diameter, was implanted in a retrograde manner. The femora implanted with titanium rods fixed with bone cements were kept in an incubator at 37 °C for 24 hr before biomechanical testing. The pullout test was conducted at a displacement rate of 0.25 mm/min to failure. Pull-out strength was calculated by dividing the force at the point of failure by the surface area of the implant in the femur.

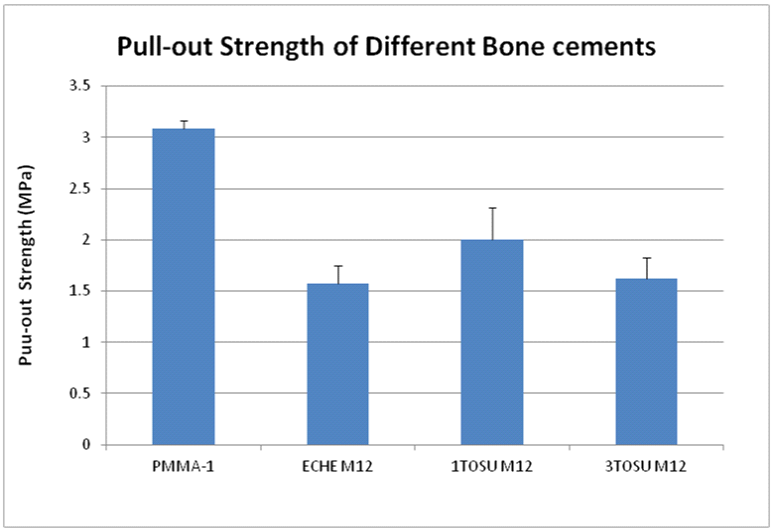


Figure 3. Pull-out strength (in MPa) using the rat femur ex vivo test.

The results of this test show that the experimental silorane bone cements do not provide equivalent mean pull-out strength as the commercial bone cement (PMMA-1). However, some of the implants secured with silorane samples did meet or exceed the mean pull-out strength of the commercial bone cement. For example, one of the femora prepared with the silorane bone cement containing 1TOSU modified M12 glass filler had a pull-out strength of 3.17 MPa. These results suggest that the silorane bone cement are capable of providing high pull-out strength, however adjustments are needed (mixing protocol, placement of cement in bone, etc…) to improve the reproducibility of these results.

Experiment 2. Rat femur ex vivo pull out test. The biomechanical examination was performed with the silorane-based bone cement including 1 TOSU coated old DY5 ("Mod old DY5" for P2_oldDY5_mod_1TOSU_B), new DY5 ("Mod new DY5" for P2_newDY5_mod_1TOSU_A) and M12 ("Mod M12" for P2_M12_mod_1TOSU_E), glass fillers. The bone cements contain 1.64% LIS Light initiation system (1.19%PIH; 0.39%CQ; 0.06% EDMAB); 37.90% SilMix; 60% glass fillers; 0.46% LMC except 1 TOSU coated new DY5 cement containing 0.8% LMC. The commercial bone cement Simplex P (Stryker) was used as control. Twenty femurs were excised from sacrificed 6-month-old rats. The femoral intramedullary canal was disrupted through femoral intercondylar notch, filled with different bone cements, respectively, and inserted with a titanium rod of 22 mm long, 1.5 mm diameter. The samples were kept in a humidified incubator at 37 ^{o}C for 24 hrs and tested biomechanically. The pullout test was conducted at a displacement rate of 0.25 mm/min to failure with the force in Newton. The values were calculated by dividing the force at the point of failure by the surface area of the implant in the femur. The graph and raw data are as follows.

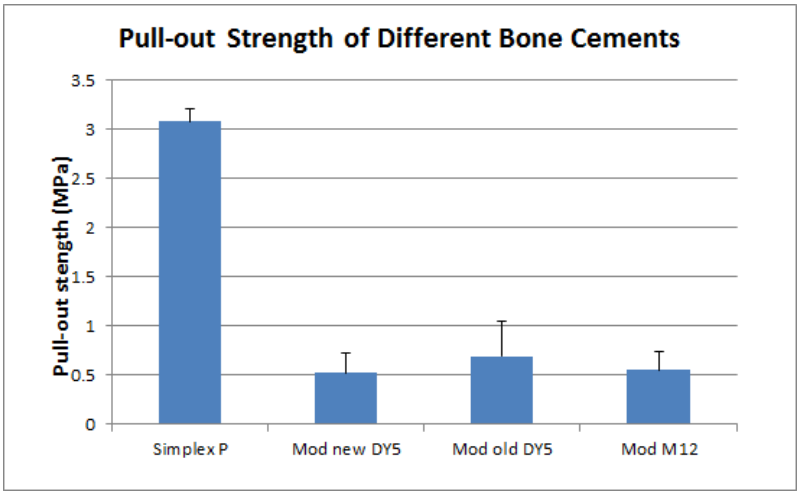


Figure 4. Pull-out strength using the rat ex vivo pull out test of three bone cements.

Sample	Simplex P	Mod new DY5	Mod old DY5	Mod M12
1	3.65	0.02	1.14	0.46
2	3.33	0.41	0.01	0.66
3	2.98	1.03	0.94	0.60
4	2.88	0.01		0.24
5	2.80	1.19		0.06
6	2.83	0.50		1.33
Mean	3.08	0.53	0.70	0.56
Standard Error	0.14	0.20	0.35	0.18

Table 2. The individual values (MPa) of Pull-out strength of different bone cements as shown in Figure 4.

Results of this study are as follows: Simplex P bone cement 3.1±0.1; 1TOSU new DY5 0.5±0.2; 1TOSU old DY5 0.6±0.2; and 1TOSU M12 0.7±0.3. All samples in Simplex P group were polymerized; two in 1TOSU new DY5; one in 1TOSU old DY5 and one in 1TOSU M12. This was very disappointing results. It appeared that there was a problem with catalyst which has since been corrected due to our quality control approach.

Experiment 3. Pull out bone mimic test. We developed a pull-out bone mimic test because of the expense and availability of rat femurs. This mimic test is performed before testing in excised rat femurs. The test is described below: Plastic tubing of 3 mm in diameter was used and cut in 30 mm length. The plastic tubes were drilled with holes of 1 mm in diameter so as to interlock the bone cements and then embedded in dental acrylic. The holder for the acrylic was a lower part of a 15 mL centrifuge tube which was cut at 3 mL and filled with 3 gram of dental acrylic and was attached to an eye hook at bottom. The plastic tubes were filled with the different bone cements and inserted with a titanium rod of 22 mm long and 1.5 mm in diameter, respectively. The samples were kept in a humidified incubator for 24 hr and the pull-out strength was determined. The formulations are shown in Table 3.

Sample ID	%SM	%PIH	%CPQ	%EDMAB	%Filler	%LMC	Modification
newP2_M12_mod_ECHE	38.03	1.19	0.40	0.06	60.00	0.32	ECHE
newP2_M12_mod_1TOSU	38.03	1.19	0.40	0.06	60.00	0.32	1TOSU
newP2_M12_mod_3TOSU	38.03	1.19	0.40	0.06	60.00	0.32	3TOSU
newP2_DY5_mod_ECHE	38.03	1.19	0.40	0.06	60.00	0.32	ECHE
newP2_M12_mod_3TOSU	38.03	1.19	0.40	0.06	60.00	0.32	1TOSU
Commercial Bone Cement, Simplex P	N/A	N/A	N/A	N/A	N/A	N/A	NA

Table 3. Composition of different bone cements

The first set of experiments was performed to evaluate pull-out strength of different bone cements with a mimic system using plastic tubing of 3 mm diameter with holes, filled with bone cement. The commercial bone cement Simplex P (Stryker) was used as the positive control. The pull-out strength values of silorane-based cements were similar or greater than that of Simplex P. There were no statistically significant differences among Simplex P, M12-ECHE, M12-1TOSU, DY5-ECHE and DY5-1TOSU. The results are shown in Figure 5. There were no statistically significant differences between Simplex P and silorane-based bone cements.

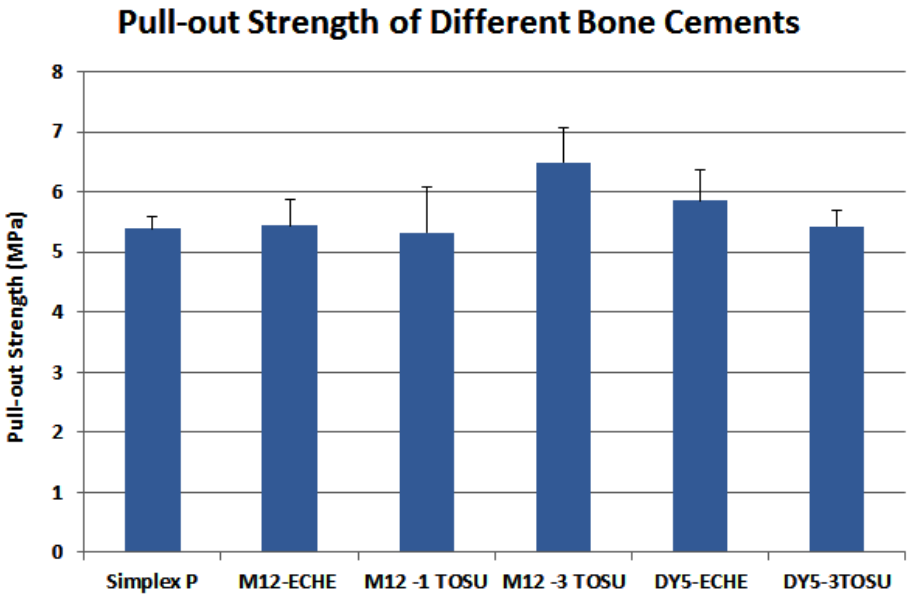


Figure 5. Pull-out strength (MPa) of different bone cements using a mimic pull-out system. There are no statistically significant differences between groups.

Experiment 4. Rat femur ex vivo pull out test. In a dry environment, the bone mimic pull out test, each of the silorane bone cements are equivalent to commercially available cement with regards to pull out strength as shown in Figure 5. A second set of experiments was performed to evaluate the different bone cements in a 'wet' environment, using excised rat femurs. The formulations of the different bone cements for the *ex vivo* pull-out assay are listed in Table 4.

Sample ID	%SM	%PIH	%CPQ	%EDMAB	%Filler	%LMC	Modification
newP2_M12_mod_ECHE	38.03	1.19	0.40	0.06	60.00	0.32	ECHE
newP2_oldDY5_mod_ECHE	38.03	1.19	0.40	0.06	60.00	0.32	ECHE
Commercial Bone Cement, Simplex P	N/A	N/A	N/A	N/A	N/A	N/A	NA

Table 4. The composition of different bone cements

Eighteen femurs were excised from sacrificed 6-month-old rats. The femoral intramedullary canal was disrupted, filled with Simplex P, silorane-based M12-ECHE or DY5-ECHE cement respectively, and inserted with a titanium rod of 22 mm long, 1.5 mm diameter. The samples were kept in a humidified incubator at 37 ^{o}C for 24 hr and tested for pull-out strength. The results for simplex P was 3.03±0.19 MPa, 1.85±0.28 Mpa for M12-ECHE and 1.90±0.39 Mpa for DY5-ECHE cement. Although the values for M12-ECHE and DY5-ECHE were close to that for Simplex P, there were statistically significant differences between Simplex P and DY5-ECHE, M12-ECHE (*P<0.05). Figure 6 shows the results.

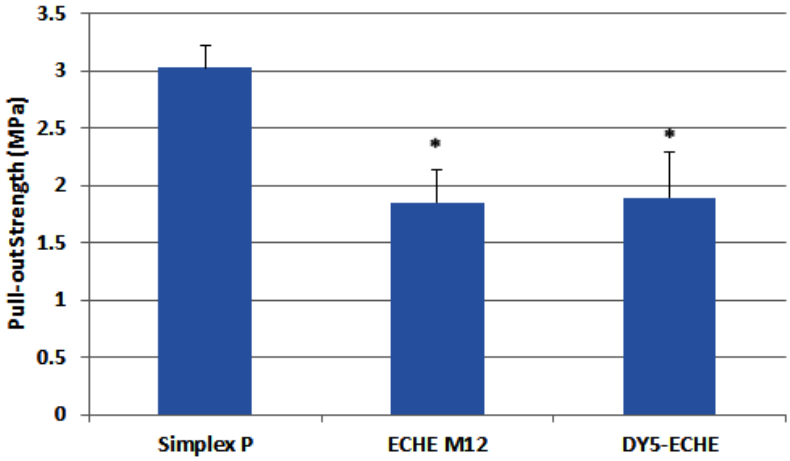


Figure 6A. Pull-out strength (MPa) of different bone cements using excised rat femora.

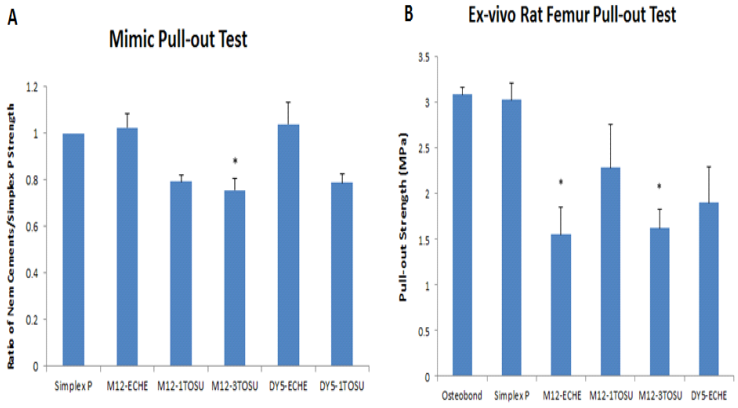


Figure 6B. Combined with data from previous year with data from Figure 6A and Figure 4. (The experiment where the catalyst did not work was excluded.) Pull-out strength (MPa) of different bone cements using excised rat femora. There was no significant difference beween the M12-1TOSU and the DY5-ECHE compared to the commercially available bone cements. *p<0.05 compared to Osteobond and Simplex P

D). Use of addends to improve pull-out strength. An investigation was initiated into the addition of addends to the cement prototypes for the improvement of pull-out strength in a wet environment. There are three addends under investigation. Two are commercially available tetraallylsilane (**A**) and 4-vinyl cyclohexyl oxirane (**C**). The third one is tris[2-(3{7-oxabicyclo[4.1.0]heptyl})ethyl] phenyl silane (**B**). It is a derivative of one of the co-monomers of the silorane bone cement, where an additional 4-vinyl

cyclohexyl oxirane is substituted for the methyl group. These two addends were chosen in order to improve crosslink density, which generally results in improved modulus, and tensile and flexural strengths, while retaining the overall biocompatibility. The chemical structures for these materials are shown in Figure 7. These experiments are ongoing.

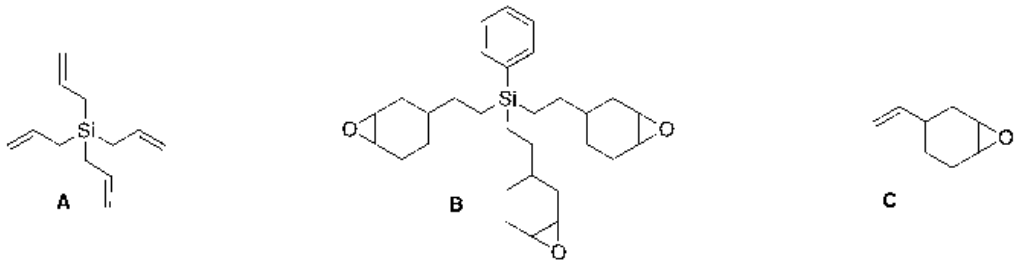


Figure 7. Structures of tetraallylsilane (**A**) and tris[2-(3{7-oxabicyclo[4.1.0]heptyl})ethyl] phenyl silane (**B**), and 4-vinyl-cyclohexene-1,2-epoxide (**C**).

FY10 Task 2: Determine the biocompatibility properties and wear debris generation of silorane bone cement prototype, Subtask 2a. Determine biocompatibility of the optimized chemically initiated silorane bone cement identified in Specific Aim 1 with relevant cell lines (i.e., MLO-A5, MSCs, L929, and HUVEC). Months 1-24.

A). Biocompatibility of Prototype Bone Cements.

Experiment 1. We tested the biocompatibility of the dual cured silorane bone cement composition that includes the TOSU surface treated glass fillers. To determine if these composites had any effect on bone cell viability, experiments were performed maintaining all parameters (1.63% LIS Light initiation system (3%PIH; 1%CQ; 0.15% EDMAB); 37.91% SilMix; 60% M12; 0.46% LMC) except that 1TOSU and 3TOSU surface treatments were used to coat M12 glass. Solid discs of the composites (0.5 mm thick x 9 mm diameter) were prepared on the day before the assay, dark cured for 12 hours, sterilized under UV, and then transferred to 48-well plates which were later seeded with MLO-A5 bone cells. Controls included neat light-cured SilMix and commercial bone cement. Results of this study are shown in Figure 8.

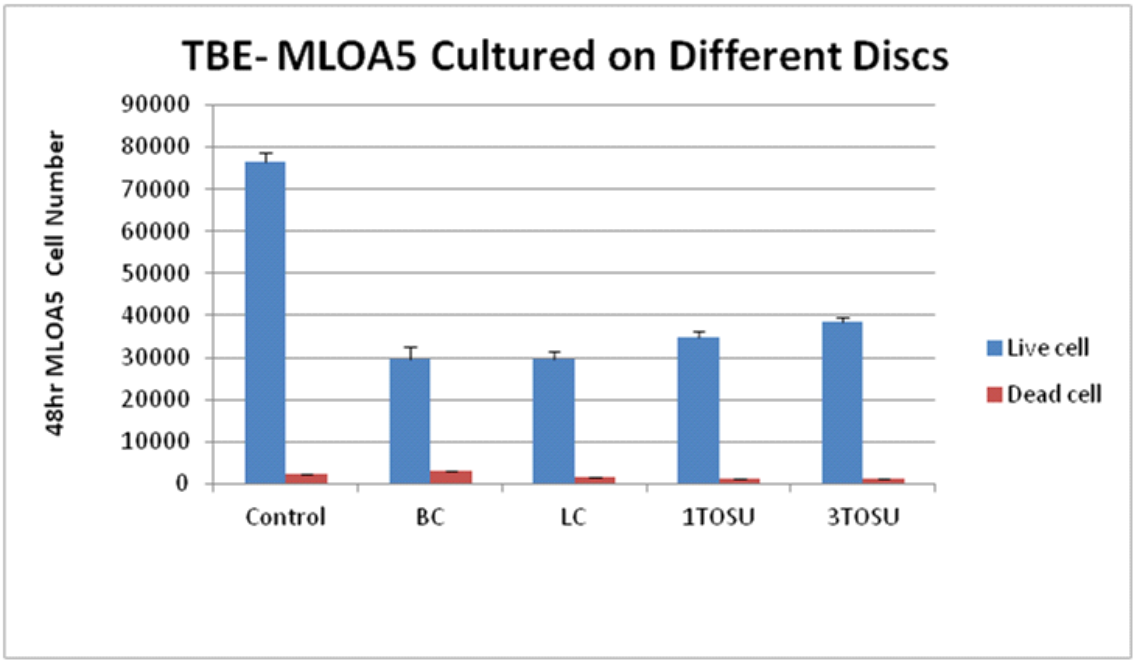


Figure 8. Viability of bone cells cultured on cement discs (percent dead cells): Zimmer® Osteobond bone cement 8.9±1.2; neat light-cured SilMix 4.0±0.5; 1TOSU 3.5±0.8; 3TOSU 3.2±0.79; showing no significant differences between the chemically cured composites containing the TOSUs as compared to light cured SilMix. The commercially available bone cement was twice as toxic as the siloranes

Experiment 2. The cytotoxicity of leachables from the same 4 composites (PMMA commercial bone cement, light cured silorane composite, dual cured silorane with 1TOSU treated M12 fillers, and dual cured silorane with 3TOSU treated M12 fillers) were also tested. Discs of each composite were prepared as before and sterilized with UV treatment. Media was conditioned on the discs for 48 hours in 48-well plates. MLO-A5 cells were plated in regular media for 48 hours before being exposed to the conditioned media for 24 hours. The cells were then tested by MTT assay Figure 9.

The results below (giving in OD readings) show that the cell activity was slightly decreased by the leachables from the 1TOSU and 3TOSU containing glasses. However, while this difference is statistically significant, all of the composites tested showed minimal cytotoxicity.

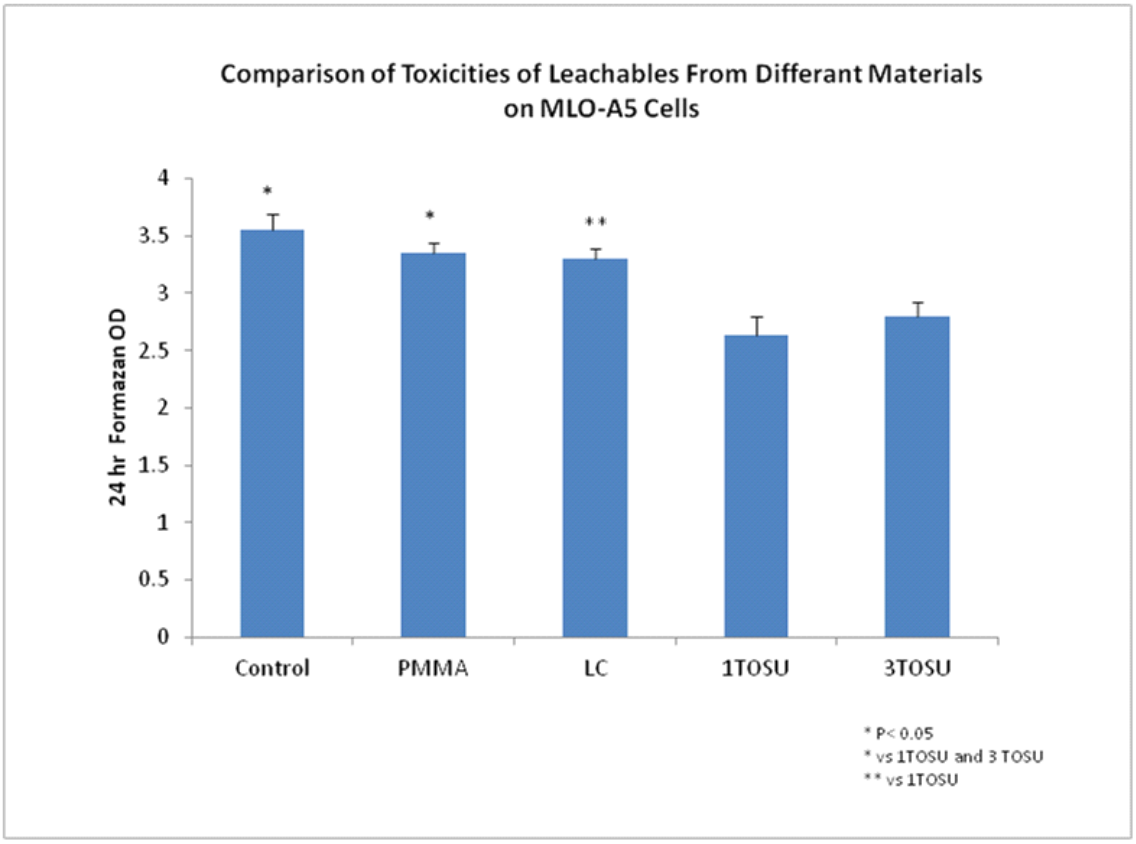


Figure 9. Comparison of toxicities of leachables from different materials on MLO-A5 cells.

Experiment 3. The biocompatibility of the dual cured silorane bone cement composition that includes the TOSU surface treated M12 glass fillers was tested. To determine if these composites had any effect on bone cell viability, experiments were performed maintaining all parameters (1.64% LIS Light initiation system (1.19%PIH; 0.39%CQ; 0.06% EDMAB); 37.90% SilMix; 60% M12; 0.46% LMC) except that 1TOSU and 3TOSU surface treatments were used to coat M12 glass. Solid discs of the composites (0.5 mm thick x 9 mm diameter) were prepared on the day before the assay, dark cured for 12 hours, sterilized under UV light, and then transferred to 48-well plates which were later seeded with MLO-A5 bone cells. Controls included neat light-cured SilMix and commercial bone cement.

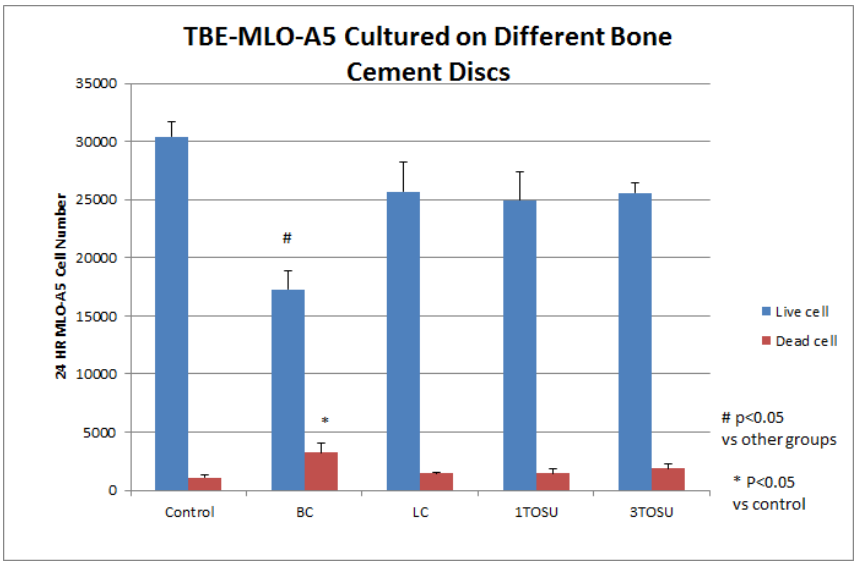


Figure 10A. 24 hr total cell number (live and dead) to assess bone cell viability on cement discs. TBE=trypan blue assay, BC=commercial bone cement; LC=light cured SilMix, 1TOSU=inclusion of 1TOSU coated M12 glass filler; 3TOSU=3TOSU coated M12 glass filler.

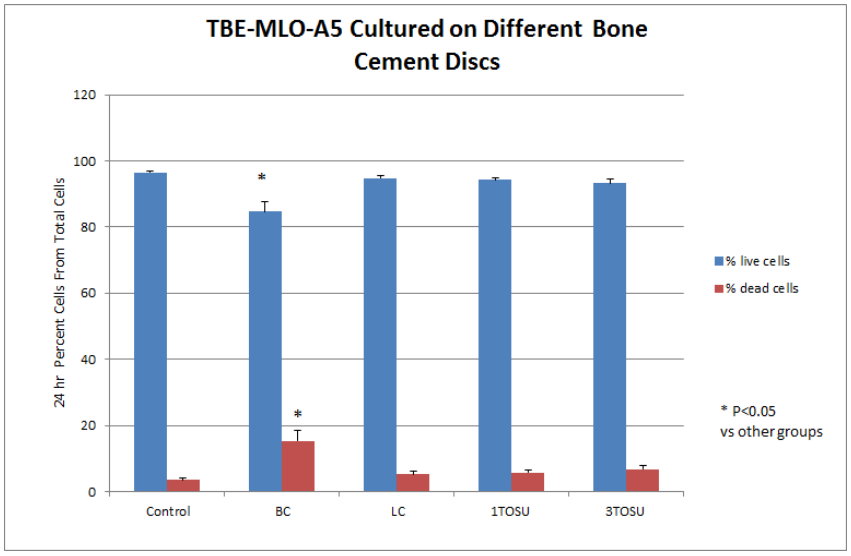


Figure 10B. 24-hour percent (live and dead) cells for different formulations. TBE=trypan blue assay, BC=commercial bone cement; LC=light cured SilMix, 1TOSU=inclusion of 1TOSU coated M12 glass filler; 3TOSU=3TOSU coasted M12 glass filler. Dead cells: Zimmer®Osteobond bone cement 18.4±4.3; neat light-cured SilMix 4.4±1.8; 1TOSU 6.3±1.6; and 3TOSU 10.6±1.8.

There were no significant differences between the chemically cured composites containing the TOSUs as compared to light cured SilMix. The commercially available bone cement was 2-3 times more toxic than the silorane formulations.

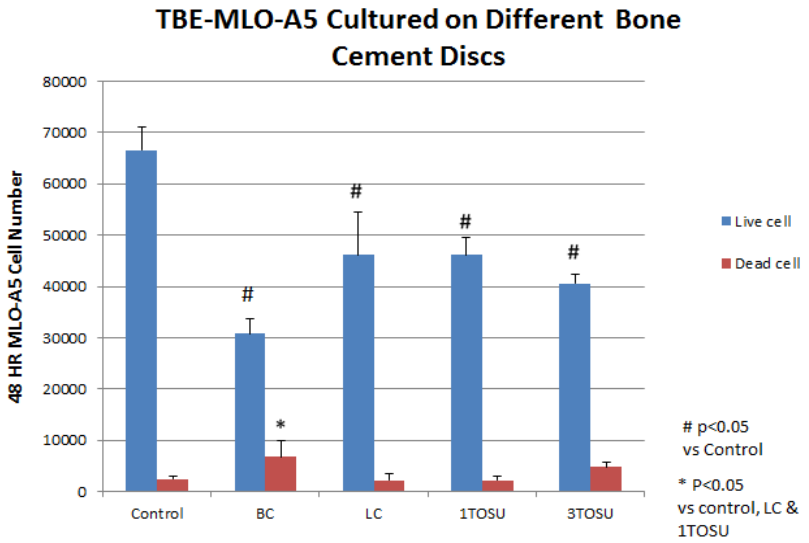


Figure 10C. Same groups as in Figures 10A and B but total cell number after 43 hours of incubation with the cement discs.

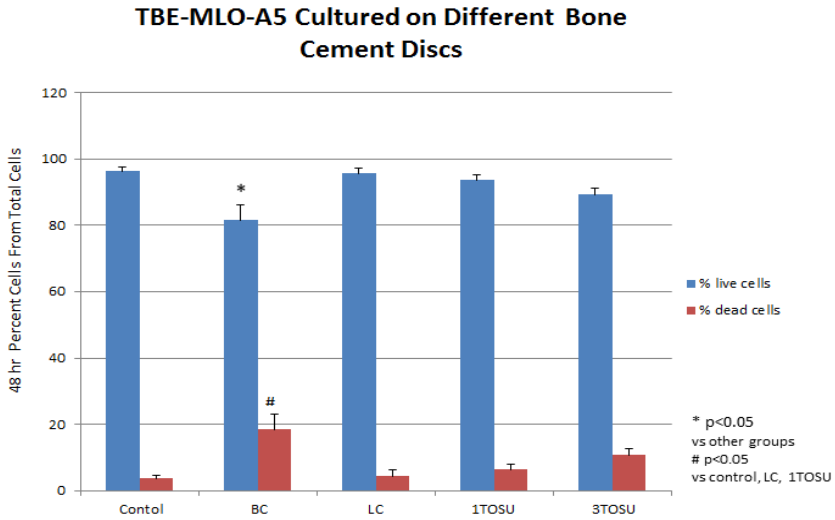


Figure 10D. Same assay as Figures 10 A, B, and C but data shown as percent live and dead total cells for each formulation. Clearly the commercially available bone cement has greater toxicity than the silorane cements.

Experiment 4. This experiment was performed to determine if the surface modified M12 glass in the composites had any effect on bone cell viability compared to formulations containing unmodified M12 glass. Experiments were performed maintaining all parameters (1.64% LIS Light initiation system (1.19%PIH; 0.39%CQ; 0.06% EDMAB); 37.90% SilMix; 60% M12; 0.46% LMC) with the glass being unmodified or surfaced treated with either 1TOSU or 3TOSU. Solid discs of the composites (0.5 mm thick x 9 mm diameter) were prepared on the day before the assay, dark cured for 12 hours, sterilized under UV light, and then transferred to 48-well plates which were later seeded with MLO-A5 bone cells. The controls were the neat light-cured SilMix, empty well control, and two commercial bone cements.

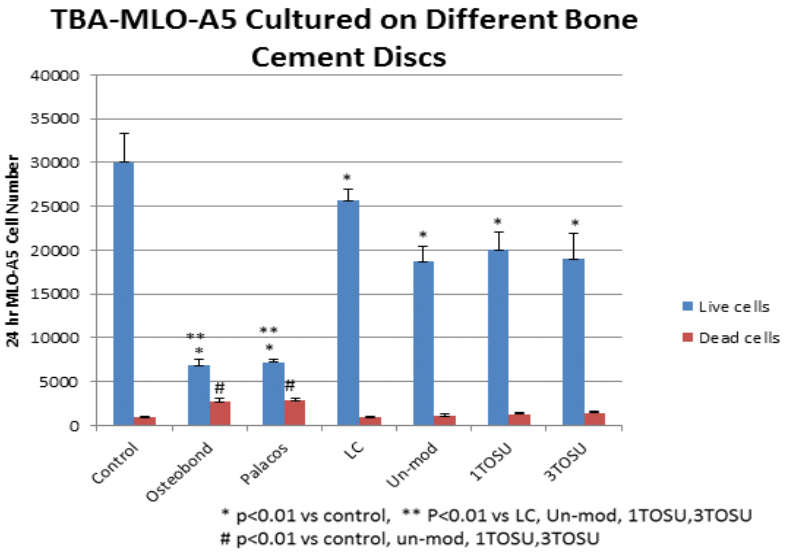


Figure 11A. 24 hour total cell numbers (live and dead) cell numbers for the different bone cement formulations.

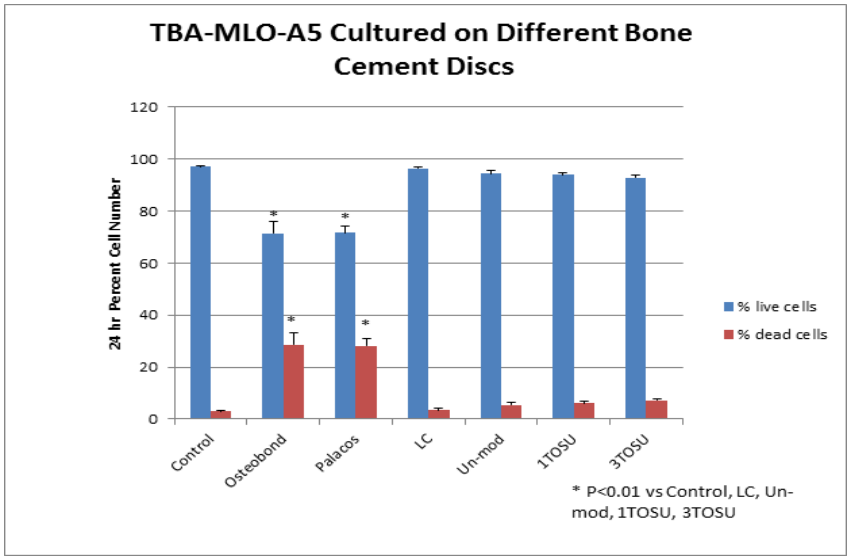


Figure 11B. Same experiment as Figure 11A, but show as percent live and dead cells for different formulations

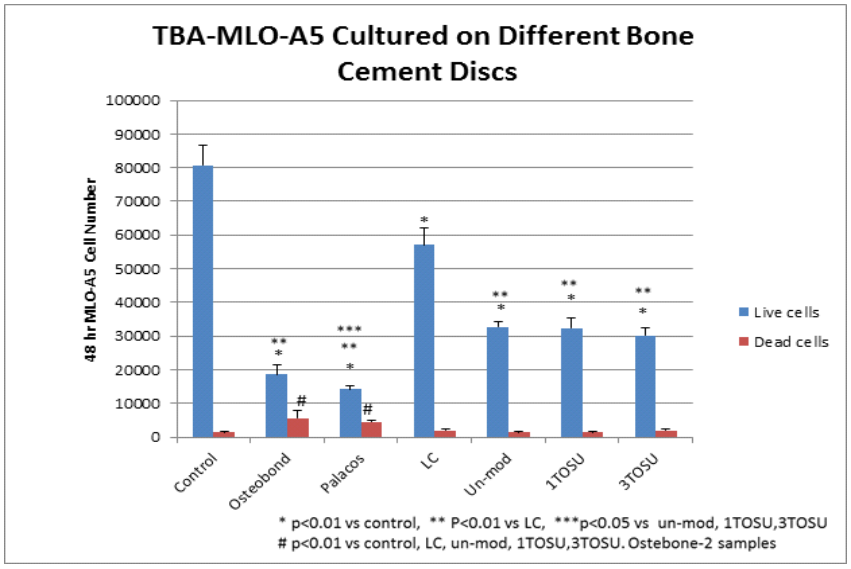


Figure 11C. 48 hour live and dead cell numbers for different bone cement formulations

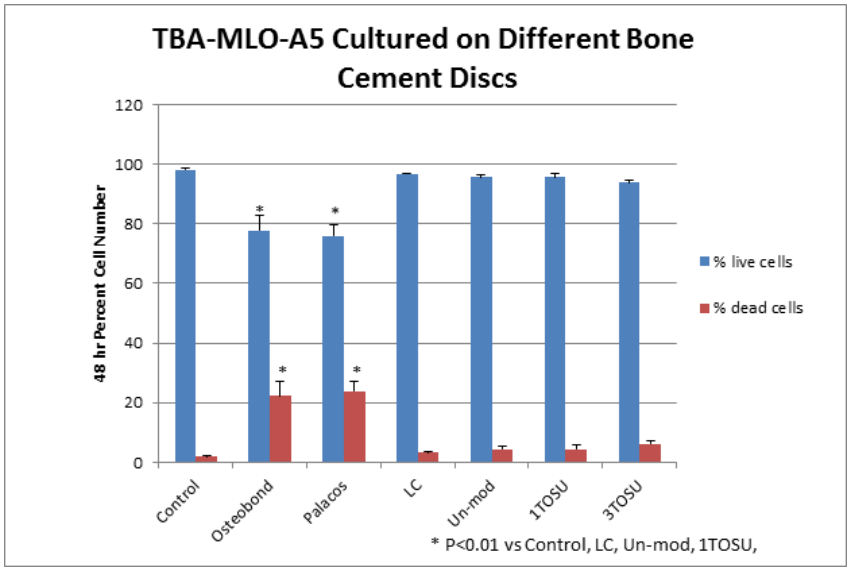


Figure 11D. 48 hour percent live and dead for the different formulations. Results of this study are as follows (percent dead cells): Zimmer®Osteobond bone cement 22.2±5.0; Zimmer®Palacos 21.5±1.5; neat light-cured SilMix 3.2±0.3; Un-mod 4.3±0.9; 1TOSU 4.3±1.2; and 3TOSU 6.2±0.77.

There were no significant differences between the chemically cured composites containing the TOSUs as compared to unmodified glass and as compared to light cured SilMix. The commercially available bone cement was 3-5 times more toxic than the silorane formulations.

Experiment 5. In last quarter we have tested the biocompatibility of the dual cured silorane-based bone cements w/wo 1TOSU surface treated glass fillers. In the first study, MLO-A5 osteoblast cell viability cultured on silorane prototype bone cement discs including 1 TOSU coated old DY5 glass fillers (Mod-old DY5), uncoated old DY5 (Un-mod old DY5) and M12 glass fillers (Un-mod M12) was tested. Light cured neat silorane (LC) discs which we have shown previously to have no toxic effects was used as a positive control along with the standard tissue culture plastic surface (Control). The bone cements contain

1.64% LIS Light initiation system (1.19%PIH; 0.39%CQ; 0.06% EDMAB); 37.90% SilMix; 60% glass fillers; and 0.46% LMC.

Solid discs of the composites (0.5 mm thick x 9 mm diameter) were prepared on the day before cell culture, dark cured for 12 hours, sterilized under UV light, and then transferred to 48-well plates before seeding with MLO-A5 cells at a density of 3.5x104 cells/cm2 in a-MEM containing 5% fetal bovine serum (FBS) and 5% calf serum (CS) at 37°C/5% CO2. The cells were maintained for 24 hrs and 48hrs. The live cells and dead cells were counted using the Trypan blue dye exclusion assay. There is a greater number of cells on the optimized tissue culture plastic compared to the resin discs. This most likely is due to reduced cell adherence to the disc surface as we have shown previously.

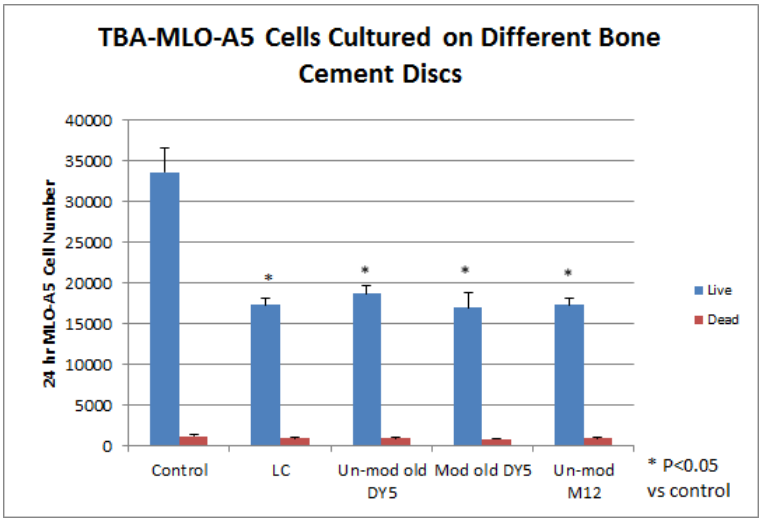


Figure 12A. The live and dead cell number after 24 hr of culture.

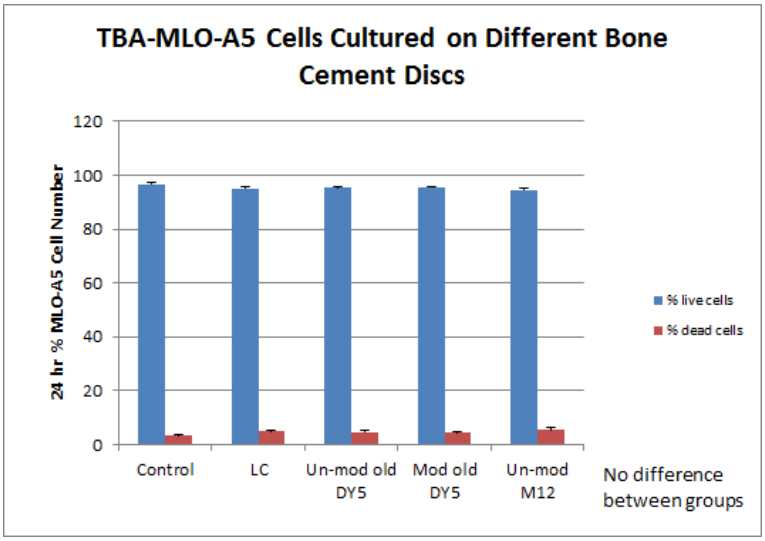


Figure 12B. The percent live and dead cell number after 24 hr of culture. Neat light-cured SilMix 4.8±042; Mod-DY5 4.42±0.24; Un-mod DY5 4.7±0.48, and Un-mod M12 5.4±0.79.

There were no significant differences between the dual cured composites w/wo 1 TOSU (un-modified/modified) and light cured SilMix.

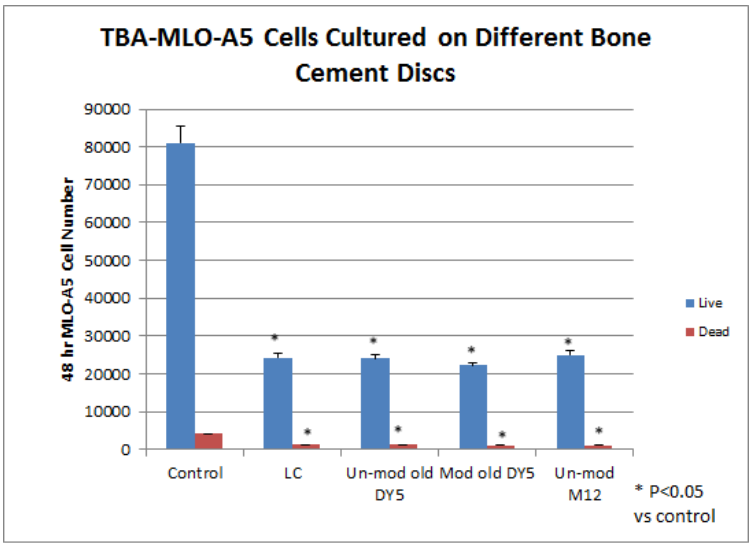


Figure 12C. The live and dead cell number after 48 hrs. of culture.

The fewer cell numbers on the resin discs is most likely due to reduced cell adherence.

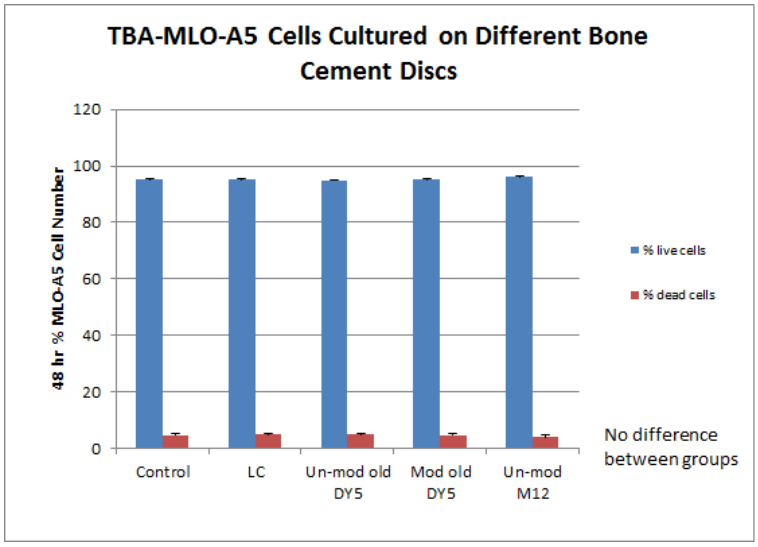


Figure 12D. The percent live and dead cell number after 48 hrs. of culture.

Again there are no significant different between the prototypes and the controls.

Experiment 6. Cell viability was examined with the silorane bone cement discs including 1 TOSU coated old DY5 (Mod old DY5), new DY5 (Mod newly synthesized DY5), and M12 (Mod M12) glass fillers, uncoated new DY5 (Un-Mod newly synthesized DY5), glass fillers. It was important to insure that the newly synthesized DY% had similar properties to the previously synthesized batch. The bone cements contain 1.64% LIS Light initiation system (1.19%PIH; 0.39%CQ; 0.06% EDMAB); 37.90% SilMix; 60% glass fillers; 0.46% LMC except 1 TOSU coated new DY5 cement containing 0.8% LMC. Solid discs of the composites (0.5 mm thick x 9 mm diameter) were prepared on the day before the assay, dark cured for

12 hours, sterilized under UV light, and then transferred to 48-well plates which were later seeded with MLO-A5 cells at a density of 3.5x104 cells/cm^2 in a-MEM containing 5% fetal bovine serum (FBS) and 5% calf serum (CS) at 37°C/5% CO2. The cells were maintained for 24 hrs and 48 hrs. The commercial bone cement Simplex P (Stryker) was used as control.

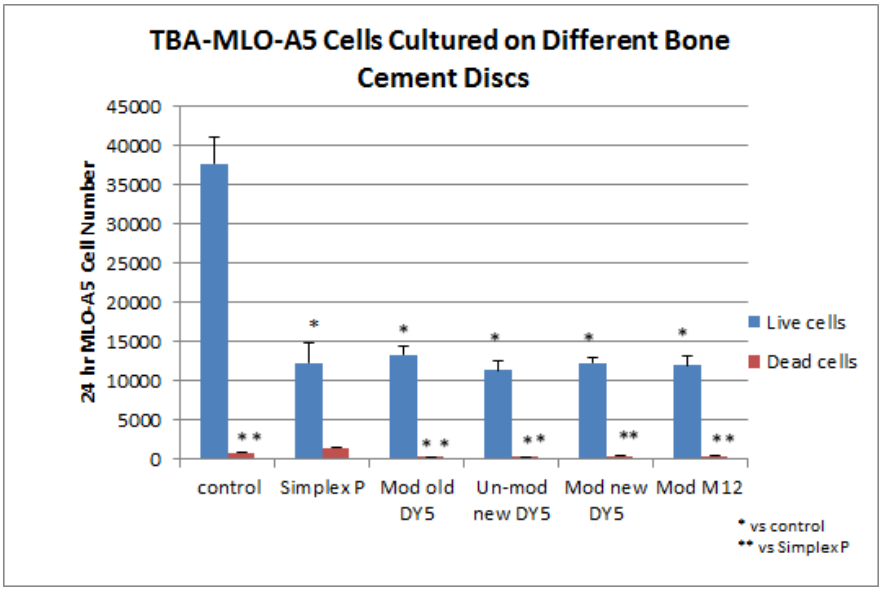


Figure 13A. The live and dead cell number after 24 hrs of culture

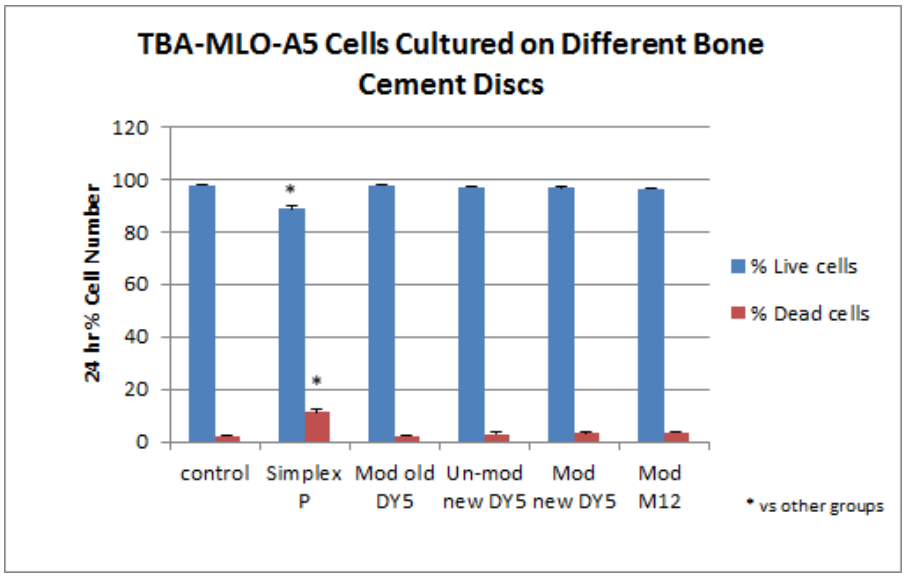


Figure 13B. The percent live and dead cell number after 24 hrs of culture.

Results of this study are as follows (percent dead cells): Simplex P bone cement 11.2±1.38; old 1TOSU DY5 2.1±0.35; Un-mod new DY5 2.9±0.36; 1TOSU new DY5 3.1±0.66; and 1TOSU M12 3.5±0.25; showing no significant differences between the dual cured composites containing 1TOSU. The commercially available bone cement was 3-5 times as toxic as the siloranes.

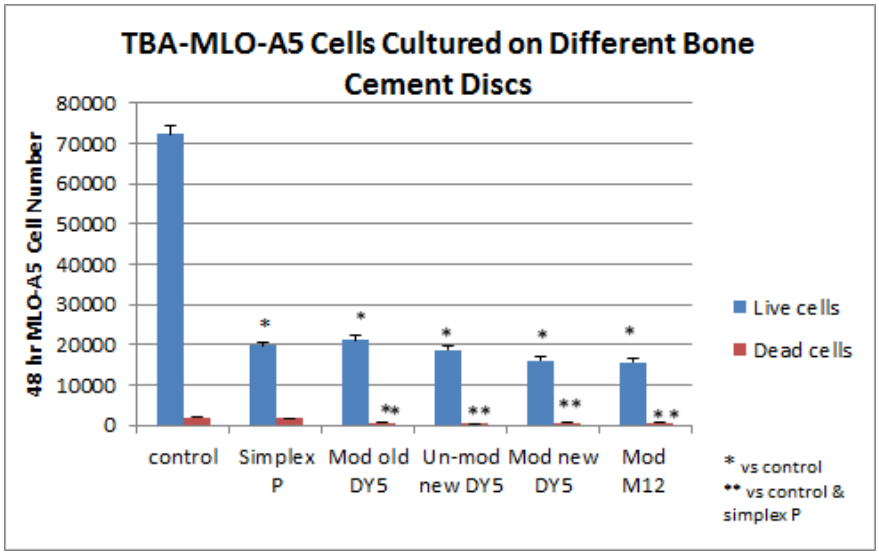


Figure 13C. The live and dead cell number after 48 hrs of culture

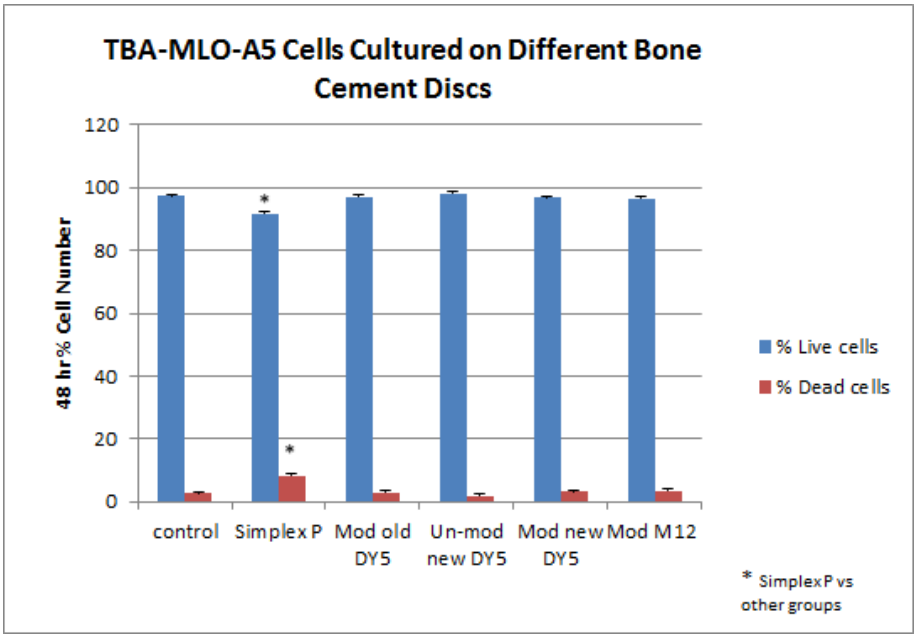


Figure 13D. The percent live and dead cell number after 48 hrs. of culture. There were no significant differences between the plastic culture surface control and the silorane prototype bone cement discs. There was significantly more cell death with the Simplex P commercially available bone cement.

Experiment 7. We have also evaluated the biocompatibility of silorane-based bone cements with ECHE-surface treated M12 (M12-ECHE) or DY5 (DY5-ECHE) glass fillers. The commercial bone cement Simplex P (Stryker) was used as the positive control. The solid discs of the composites (0.5 mm thick x 9 mm diameter) were prepared on the day before cell culture, dark cured for 12 hours, sterilized under UV light, and then transferred to 48-well plates before seeding with MLO-A5 cells at a density of 3.5x104 cells/cm2 in a-MEM containing 5% fetal bovine serum (FBS) and 5% calf serum (CS) at 37°C/5% CO2. The cells were maintained for 24 hrs and 48hrs. The live cells and dead cells were counted using the Trypan blue dye exclusion assay. The percent live and dead cells were calculated. Results of this study are as follows (percent dead cells): Simplex P 16.61±0.56; M12-ECHE 4.49±0.47; DY5-ECHE

4.47±0.46. The commercially available bone cement was 3 times as toxic as the silorane cements. Table 5 shows compositions of the different cements and figures 14 and 15 show live and dead cell numbers on the different discs.

Sample ID	%SM	%PIH	%CPQ	%EDMAB	%Filler	%LMC	Modification
newP2_M12_mod_ECHE	38.03	1.19	0.40	0.06	60.00	0.32	ECHE
newP2_DY5_mod_ECHE	38.03	1.19	0.40	0.06	60.00	0.32	ECHE
Commercial Bone Cement, Simplex P	N/A	N/A	N/A	N/A	N/A	N/A	NA

Table 5. The composition of different bone cements.

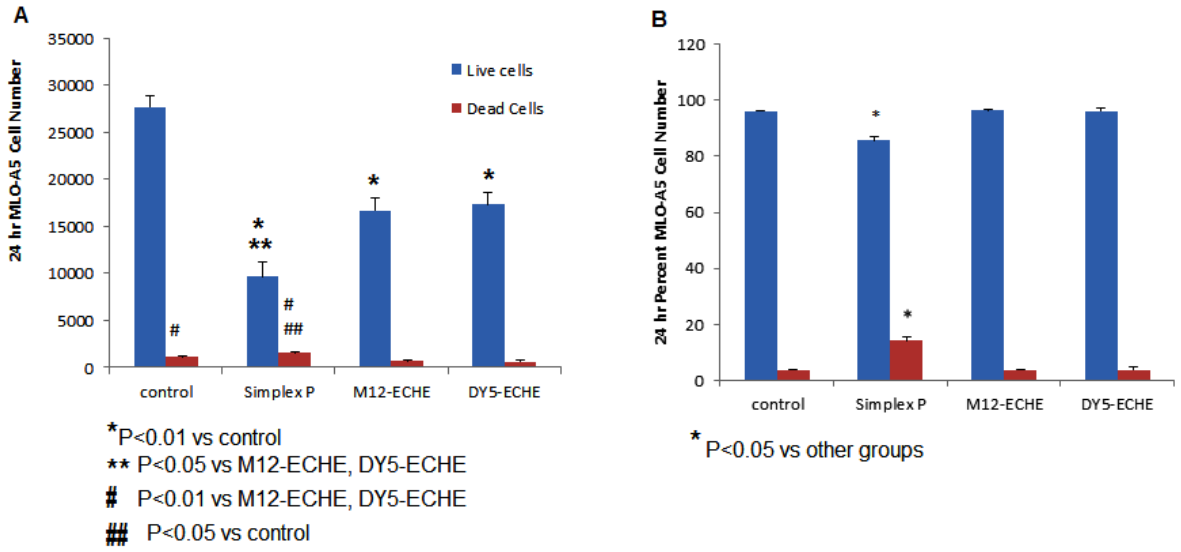


Figure 14. MLO-A5 cells were cultured on different bone cements discs after 24 hr of culture. (A). the live and dead cell number. (B). the percent live and dead cell number.

There were significant differences in percent dead cell number between the Simplex P and silorane-based bone cements ($P<0.05$).

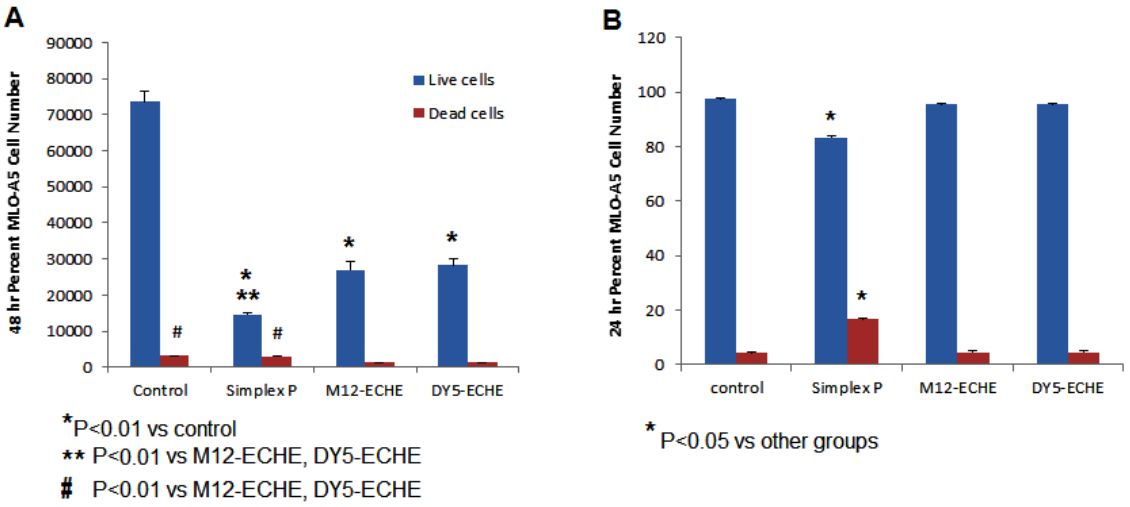


Figure 15. MLO-A5 cells were cultured on different bone cements discs after 48 hr of culture. (A). The live and dead cell number and (B) the percent live and dead cell number. There were significant differences in percent dead cell number between the Simplex P and silorane-based bone cements (P<0.05).

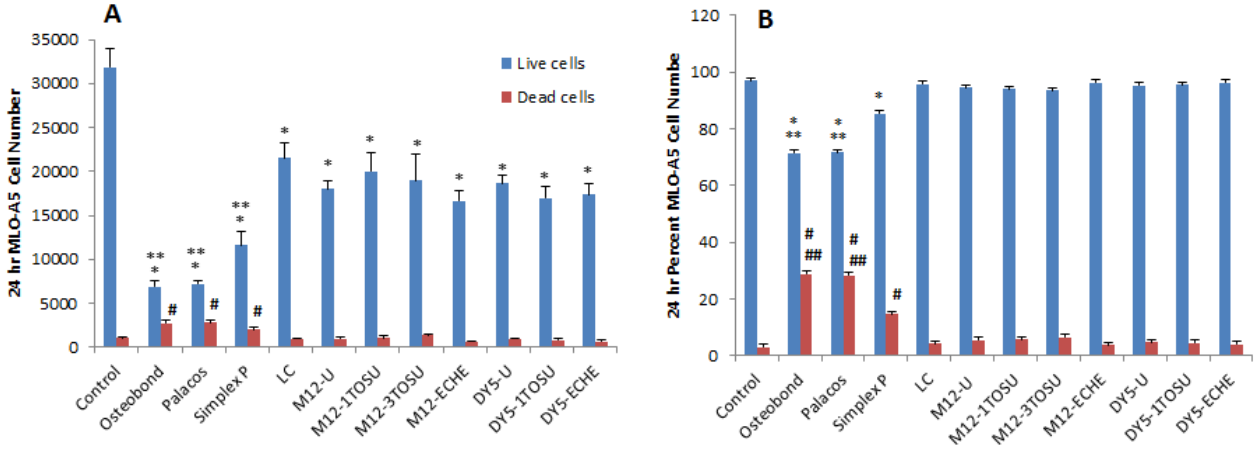


Figure 16. Combination of all of the above data. MLO-A5 bone cells cultured for 24 hrs on discs for determination of viability using the trypan blue exclusion assay. (A). Total number of live and dead cells. (B) Percent live or dead cells. A. *P<0.01 vs control; **P<0.05 vs LC, M12-U,M12-1TOSU, M12-1TOSU, DY5-1TOSU and DY5-ECHE; #P<0.05 vs control, M12-U,M12-1TOSU, M12-1TOSU, M12-ECHE, DY5-1TOSU and DY5-ECHE. B. *P<0.05 vs control, M12-U,M12-1TOSU, M12-1TOSU, M12-ECHE, DY5-1TOSU and DY5-ECHE; **P<0.05 vs Simplex P; # P<0.05 vs control, M12-U,M12-1TOSU, M12-1TOSU, M12-ECHE, DY5-1TOSU and DY5-ECHE; ## p<0.05 vs Simplex P

B). Testing for potential osteogenic properties. An experiment was performed to determine whether the silorane-based bone cements are osteogenic. The silorane-based bone cements with unmodified (DY5-U), ECHE (DY5-ECHE) or 1TOSU-surface treated DY5 (DY5-1TOSU) glass fillers were investigated. The commercial bone cement Simplex P (Stryker) was used as the positive control. The solid discs of the composites (0.5 mm thick x 9 mm diameter) were prepared, sterilized under UV light, and then transferred to 48-well plates. The MLO-A5 cells were seeded on the different cement discs at a density of 3.5x104 cells/cm2 in a-MEM containing 5% fetal bovine serum (FBS) and 5% calf serum (CS) at 37°C/5% CO2, and cultured until confluence. At confluence (approximately 2 days) after plating (day 0),

the culturing medium was replaced with mineralization medium. The mineralization medium was replaced every 2 days. After 6 days in mineralizing medium, the levels of alkaline phosphatase were examined. There were statistically significant differences in alkaline phosphatase levels between the DY5-U ($p < 0.001$), the DY5-1TOSU ($p < 0.01$), the DY5-ECHE ($P < 0.05$) and the control. There were also significantly higher amounts of alkaline phosphatase found in all 3 silorane bone cements cultures compared to Simplex P ($P < 0.001$). The results strongly indicate that the silorane-based bone cements with glass fillers are promising materials for anchoring implants and enhancing osseointegration. Table 4 shows the composition of the different cements and figure 17 shows alkaline phosphatase activities of MLO-A5 cells on the discs.

Sample ID	%SM	%PIH	%CPQ	%EDMAB	%Filler	%LMC	Modification
newP2_DY5_unmod	38.03	1.19	0.40	0.06	60.00	0.32	None
newP2_DY5_mod_1TOSU	38.03	1.19	0.40	0.06	60.00	0.32	1TOSU
newP2_DY5_mod_ECHE	38.03	1.19	0.40	0.06	60.00	0.32	ECHE
Commercial Bone Cement, Simplex P	N/A	N/A	N/A	N/A	N/A	N/A	NA

Table 6. The composition of different bone cements

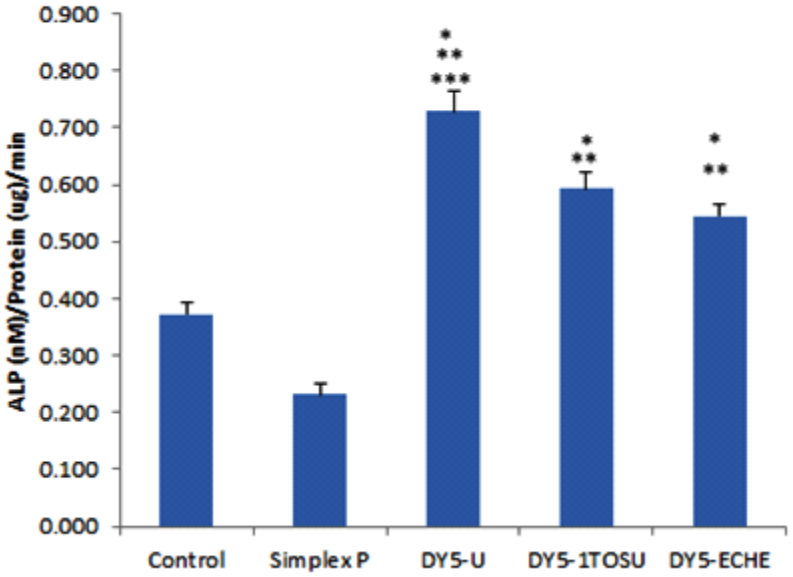


Figure 17. Alkaline phosphatase activity of MLO-A5 cells seeded on different bone cements discs after 6 days of culture. There were statistically significant differences between silorane-based bone cements and Simplex P. * compared to control, ** compared to Simplex P, *** compared to DY5-ECHE. This data suggests that the silorane bone cements may promote bone formation in vivo.

FY10 Task 3: Determine the biological response to silorane bone cement in animal models, Subtask 3a. Small Animal (Rat) Model. Months 13-18

We have ordered the rats for the in vivo experiments. We will test the top performing silorane bone cements: M12-1TOSU, M12-3TOSU, DY5-ECHE, and DY5-3TOSU.

FY10 Task 3. Determine the biological response to silorane bone cement in animal models, Subtask 3a. Large Animal (Swine) Model. Months 16-24.

The task has yet to start.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

FY10 Task 1: Develop a silorane bone cement suitable for in vivo studies and to optimize the formulation of the chemically and mixed cured cement prototypes, Subtask 1a. Silanization of filler particles. Months 1-12.

Continue treatment of glass filler for future studies. The post-doc under Dr. Schuman's direction was hired October 1st to conduct this aspect of the study.

FY10 Task 1: Develop a silorane bone cement suitable for in vivo studies and to optimize the formulation of the chemically and mixed cured cement prototypes, Subtask 1b. Optimize composite formulation with respect to mechanical/handling properties. Months 13-24.

Optimize mixing protocol to improve consistency of bone cement. Bone cement consistency will be determined through mechanical testing (flexural strength and modulus).

FY10 Task 2: Determine the biocompatibility properties and wear debris generation of silorane bone cement prototype, Subtask 2a. Determine biocompatibility of the optimized chemically initiated silorane bone cement identified in Specific Aim 1 with relevant cell lines (i.e., MLO-A5, MSCs, L929, and HUVEC). Months 1-24.

Next year, we plan to perform the wear debris studies, most likely on M12-1TOSU, M12-3TOSU, DY5-ECHE and DY5-3TOSU formulations.

FY10 Task 3: Determine the biological response to silorane bone cement in animal models, Subtask 3a. Small Animal (Rat) Model. Months 13-18.

We have ordered the rats and will choose the top silorane bone cements, most likely M12-1TOSU, M12-3TOSU, DY5-ECHE, and DY5-3TOSU for in vivo testing.

FY10 Task 3: Determine the biological response to silorane bone cement in animal models, Subtask 3a. Large Animal (Swine) Model. Months 16-24.

This task will start after the rat studies including histological evaluation have been completed.

Comments on administrative and logistical matters.

Dr. Schuman has identified an appropriate post-doc, who has started as of 1 October 2012. Despite the delay in hiring research personnel, work has continued and Dr. Schuman has still been able to perform syntheses and use the products to modify the fillers. For synthesis of the surface modifiers, Drs. Schuman and Kilway (Brad Miller, a doctoral student in Dr. Kilway's lab) have made progress. Therefore, we have been able to produce surface treated glass fillers for commencing testing. Dr. Schuman is continuing synthesis of the TOSU monomers and modifying the glass fillers.

Undergraduate students, Daniel Rodman (continuing) and Grant Meyer (June – July 2012), were trained to run the mechanical testing protocols for this project. James Cash (continuing) has been working on the synthesis of SilMix.

KEY RESEARCH ACCOMPLISHMENTS:

1). We have shown that the silorane resin (Eick et al JBMR, 2012 attached) and silorane composites have significantly lower toxicity and better biocompatibility than commercially available polymer bone cements of PMMA and BisGMA-TEGDMA composites. The reduced toxicity and improved biocompatibility is attributed to the intrinsic properties of silorane and low toxicity of the selected initiation system (dual cure).

2) Monomers (TOSU ring structure, oxiranes including ECHE) have been used to modify the glass fillers. The modified surfaces provide slight volume expansion during polymerization in addition to the benefits of wetting and are self-dispersing. As a result, such surface modifications enhance the mechanical properties. In addition, the modified interface should be very stable in the human body leading to a very slow degradation of the cement. The decreased bone cement's interfacial stress may eventually lead to more stable and durable hip and knee implants.

3) The silorane bone cements also appear to support osteogenesis in contrast to commercial bone cement. The in vivo animal tests presently being performed should validate the in vitro observations. This property would prove invaluable with regards of osseointegration of the prosthetic device with bone leading to greater stability.

REPORTABLE OUTCOMES:

Manuscripts:

1) *Silorane resin supports proliferation, differentiation, and mineralization of MLO-A5 bone cells in vitro and bone formation in vivo.* J David Eick, Cielo Barragan-Adjemian, Jennifer Rosser, Jennifer R Melander, Vladimir Dusevich, Rachel A Weiler, Bradley D Miller, Kathleen V Kilway, Mark R Dallas, Lianxing Bi, Elisabet L Nalvarte, Lynda F Bonewald Journal of Biomedical Materials Research. Part B, Applied biomaterials. 04/2012; 100(3):850-61.

2) *Estimation of properties of a photoinitiated silorane-based composite with potential for orthopaedic applications.* Jennifer R Melander, Rachel A Weiler, Bradley D Miller, Thomas P Schuman, Kathleen V Kilway, Delbert E Day, Mariano Velez, J David Eick Journal of Biomedical Materials Research. Part B, Applied biomaterials. 11/2011; 100(1):163-9

Abstracts and Presentations:

Melander J. R.*; Holmes, R. R.; Weiler, R. A.; Miller, B. D.; Kilway, K. V.; Schuman, T. P.; Eick, J. D., "TOSU Addends Maintain Mechanical Properties while Decreasing Polymerization Stress," Abstract # 973, American Association for Dental Research 41st General Session, Tampa, FL, March 21-24, 2012.

Kilway, K. V.*, "Silorane Composites for Orthopaedic Applications, Part III" University of Missouri – Kansas City Center of Excellence in Mineralized Tissues Seminar Series, Kansas City, MO, June 20, 2012.

Melander J. R.*; Holmes, R. R.; Yao, X.; Weiler, R. A.; Eick, J. D "Measuring Strain in Bone Cement with Carbon Nanotubes" abstract # SBC2012-80620, American Society of Mechanical Engineers 2012 Summer Bioengineering Conference, Fajardo, Puerto Rico, June 20-23, 2012.

Holmes, R. R.*; Melander J. R.; Weiler, R. A.; Schuman, T. P.; Kilway, K. V.; Eick, J. D "Polymerization Stress and the Influence of TOSU Addends on Methacrylate Composites" abstract # SBC2012-80627, American Society of Mechanical Engineers 2012 Summer Bioengineering Conference, Fajardo, Puerto Rico, June 20-23, 2012.

Miller BD, Weiler RA, Melander JR, Nalvarte EL, Kilway KV, Bonewald LF, and Eick JD. "Biocompatibility of a Chemically Initiated Silorane Resin." Poster Presentation, 89th Annual Meeting & Exhibition of the International Association for Dental Research, San Diego, CA, March 2011.

Weiler RA, Melander JR, Miller BD, Kilway KV, Bonewald LF, and Eick JD. "Physical Properties of Filled Chemically Initiated Silorane Biomaterials." Poster Presentation, 89th Annual Meeting & Exhibition of the International Association for Dental Research, San Diego, CA, March 2011.

Melander JR, Weiler RA, Miller BD, Kilway KV, and Eick JD. "Handling Properties and Exothermicity of Chemically Initiated Silorane Biomaterial." Poster Presentation, 89th Annual Meeting & Exhibition of the International Association for Dental Research, San Diego, CA, March 2011.

Melander JR, Weiler RA, Miller BD, Kilway KV, and Eick JD. "Flexural Properties of Silorane Bone Cement." Poster Presentation, ASME 2011 Summer Bioengineering Conference, Farmington, PA, June 2011.

Melander JR, Weiler RA, Miller BD, Kilway KV, and Eick JD. "Improving the Strength of a Silorane Bone Cement." Poster Presentation, Missouri Musculoskeletal Conference, July 2011.

Abstract submitted, accepted, and to be presented.

Kilway, K. V.*; Eick, J. D.; Bi, L.; Weiler, R. A.; Miller, B. D.; Bunnell, T. J.; Melander J. R.; Schuman, T. P.; Bonewald, L. F., "Dual-initiated Silorane Formulations for Use as a Bone Cement Alternative," Poster # 1232, Orthopaedic Research Society 2013 Annual Meeting, San Antonio, TX, January 26-29, 2013.

Presentations:

Tom Schuman, Invited speaker, "Influence of the Composite Filler-to-Matrix Interface on Bulk Properties," Missouri State University, Springfield, MO, 1 February 2012.

Licenses: A patent cooperative treaty (PCT) has been published for the innovative chemical initiator systems by UMKC and Nanova will have an exclusive license (under negotiation).

Degrees obtained that are supported by this award: James Cash, BA in Chemistry May 2012.

Funding applied for based on this work: An SBIR entitled "Development and Commercialization of a Novel Silorane Bone Cement" has been submitted and will be reviewed this month. The ultimate goal of this project is to develop an entirely new silorane bone cement with significantly improved biocompatibility and reduced toxicity, potential to support bone growth, reduced heat generation, and good handling properties for clinical uses to replace currently available PMMA bone cement. As the leading bone cement in the market, PMMA has been well accepted by orthopedists for a variety of applications due to its good handling properties and other excellent attributes. However, PMMA still suffers from many drawbacks such as poor osteointegration, blood pressure lability caused by leached

MMA monomer, and thermal tissue necrosis, which may contribute to clinical failure such as implant aseptic loosening. Our recent results demonstrated that the silorane composite has the potential to support osseous integration around the cemented total joint implant and may generate less immunogenic wear debris. We believe a more biocompatible silorane based bone cement will benefit millions of patients and will be a major contribution to healthcare in the United States. However, the silorane based bone cement, despite the very promising preliminary data, is not yet ready for the market for the following reasons: 1) The existing prototype formulation cannot be mixed uniformly in the operating room and a novel formulation needs to be developed; 2) Reliable large scale fabrication of several components is not commercially available; and 3) Large animal trials and simulated tests need to be conducted to further confirm the safety and effectiveness of the silorane based cements. Therefore, the following specific aims are proposed:

PHASE I AIM I: Identify a two-component formulation that exhibits mechanical, exothermic, and handling properties that meets the basic ISO standards.

PHASE I AIM II: Investigate the biocompatibility of the silorane cement based on the two-component formulation.

PHASE II AIM I: Scale up the raw materials production and identify the formulation ranges that exhibit optimal mechanical, exothermic, handling, drug elution, and biological properties, which meet or surpass the FDA and ISO requirements.

PHASE II AIM II: Perform simulated tests along with *ex vivo* and *in vivo* experimentation to validate safety, application design, and effectiveness.

Employment received based on experience/training supported by this award.

James Cash – Logan College of Chiropractic University Programs in St. Louis, MO

Jenny Melander- Applied to assistant professor positions in biomedical or mechanical engineering at the University of Virginia, University of Kentucky, University of Tennessee and Vanderbilt University. She has accepted a position at the University of Nebraska-Lincoln as an extension assistant professor.

CONCLUSIONS*:*

We have developed novel silorane bone cements with excellent properties that are ready for in vivo animal testing. For the coming year, silorane cements with the best properties will be tested in rats and the composition that gives the ideal pull out properties, lack of inflammation, and osseointegration will be used and tested in a swine model. While conducting the animal studies it will be determined if wear debris from these cements have any inflammatory or osteoclast activation/resorption properties. We are hopeful that this technology will soon be licensed and that the SBIR submitted for the commercialization of the silorane bone cement will be funded. With the improved biocompatibility, reduced exothermicity, good handling properties, incorporation of antibiotics/growth factors, and potential for osseointegration/osseoinduction, this material has potential to be used for screw augmentation, total hip/knee joint replacement, and other orthopedic and dental applications. The reduced curing temperature of approximately 26°C of the dual initiated silorane composite makes it possible to carry and deliver a wide range of antibiotics and potentially growth factors, which previously could not be used in PMMA bone cements. The development of the silorane bone cement is very promising.

APPENDICES:

1) Journal Article: *Silorane resin supports proliferation, differentiation, and mineralization of MLO-A5 bone cells in vitro and bone formation in vivo.*

J David Eick, Cielo Barragan-Adjemian, Jennifer Rosser, Jennifer R Melander, Vladimir Dusevich, Rachel A Weiler, Bradley D Miller, Kathleen V Kilway, Mark R Dallas, Lianxing Bi, Elisabet L

Nalvarte, Lynda F Bonewald Journal of biomedical materials research. Part B, Applied biomaterials. 04/2012; 100(3):850-61.

2) Journal Article: *Estimation of properties of a photoinitiated silorane-based composite with potential for orthopaedic applications.*
Jennifer R Melander, Rachel A Weiler, Bradley D Miller, Thomas P Schuman, Kathleen V Kilway, Delbert E Day, Mariano Velez, J David Eick Journal of biomedical materials research. Part B, Applied biomaterials. 11/2011; 100(1):163-9

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Estimation of properties of a photoinitiated silorane-based composite with potential for orthopaedic applications

Jennifer R. Melander,[1] Rachel A. Weiler,[2] Bradley D. Miller,[2] Thomas P. Schuman,[3] Kathleen V. Kilway,[2] Delbert E. Day,[4] Mariano Velez,[4] J. David Eick[1]

[1]School of Dentistry, Department of Oral Biology, University of Missouri-Kansas City, Kansas City, Missouri
[2]Department of Chemistry, University of Missouri-Kansas City, Kansas City, Missouri
[3]Department of Chemistry, University of Missouri Science and Technology, Rolla, Missouri
[4]MO-SCI Corporation, Rolla, Missouri

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Abstract: We have synthesized a filler-reinforced silorane composite that has potential applications in orthopaedic surgery, such as for a bone stabilizer. The purpose of the present work was to develop a method for estimating four properties of this material; namely, maximum exotherm temperature, flexural strength, flexural modulus, and fracture toughness. The method involved the use of mixture design-of-experiments and regression analysis of results obtained using 23 formulations of the composite. We validated the estimation method by showing that, for each of four composite formulations that were not included in the method development, the value of each of the aforementioned properties was not significantly different from that obtained experimentally. Our estimation method has the potential for use in the development of a wide range of orthopaedic materials. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 100B: 163–169, 2012.

Key Words: orthopedic biomaterials, design-of-experiments, mechanical properties, handling properties

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INTRODUCTION

There is a wide range of methods used to stabilize fractured bones, including plaster casts, splints, external fixators, and intramedullary pinning.[1] These methods are adequate for the majority of fractures, but, in some cases, such as comminuted fractures of small bones, additional methods are needed. Successful bone fracture outcomes depend on adequate stabilization during the healing process.[2–4] One of those additional methods involves the use of a bone stabilizer.[5] It has been suggested that composites, based on a silorane resin as the matrix, are potential candidates for polymeric bone stabilizers. With this type of stabilizer, the polymer is applied directly to the bone and polymerizes directly on it, thereby obviating the need for ample and healthy bone for placement of pins and/or rods, and allowing the stabilization of the fracture without joint immobilization.[6,7] There are limited data on the properties of these silorane resin-reinforced composites[8–15] due to the recent development of siloranes for dental applications.[9,16] Furthermore, novel silanized filler particles have not been explored to achieve a solid resin/filler particle interface and subsequent improved mechanical properties.[17–19] This situation may be rectified by developing validated methods for estimating their properties. In the present contribution, we give details of such a method, with reference to four properties of particular importance to materials to be used as bone stabilizers, namely, maximum exotherm temperature (T_{max}), flexural strength (σ_B), flexural modulus (E_B), and fracture toughness (K_{IC}).

MATERIALS AND METHODS

Preparation of composites

The matrix for these composites was prepared in house using chemicals obtained from Cambridge Isotopes, Gelest, Aldrich, and Alfa Aesar. The matrix was a silorane resin (SilMix, SM) that comprised 50 Wt/Wt % of (bis[2-(3{7-oxabicyclo[4.1.0]heptyl})-ethyl]methylphenyl silane) (PHEPSI) and 50 Wt/Wt % 2,4,6,8-tetrakis(2-(7-oxabicyclo[4.1.0]heptan-3-yl)ethyl)-2,4,6, 8-tetramethyl-1,3,5,7,2,4,6,8-tetraoxatetrasilocane (CYGEP; Figure 1). To formulate the composites, three different fillers were added to the matrix, with the choice of filler being based on four criteria, namely, interfacial compatibility with the matrix, low cytotoxicity, a refractive index close to that of the matrix, and no inhibition of polymerization. The fillers used were a glass, yttria alumino-silicate; 15.0 Wt % Y_2O_3, 5.0 Wt % Al_2O_3, and 80 Wt

Correspondence to: K. V. Kilway; e-mail: kilwayk@umkc.edu
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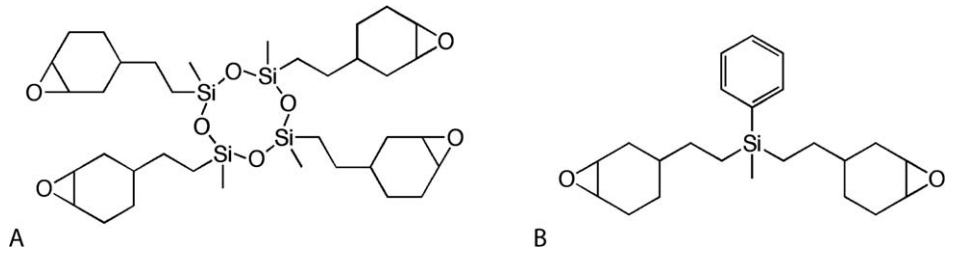

FIGURE 1. Components of silorane resin (SilMix, SM). (A) CYGEP and (B) PHEPSI. Note the epoxide groups (C–O–C) at the extremities of the structures.

% SiO_2 (DY5), another glass, barium boroaluminosilicate; 54.5 Wt % SiO_2, 5.9 Wt % Al_2O_3, 10.5 Wt % B_2O_3, and 29.1 Wt % BaO (M12), and alumina nanorods ($\sim$ 30 nm width $\times$ 450 nm length) prepared from boehmite nanorods. The surface of each of the fillers was modified with 2-(3,4-epoxycyclohexyl) ethyl trimethoxysilane (ECHE-TMS) by refluxing with 1 vol/vol % ECHE-TMS dissolved in methylisobutylketone. The composites were photoinitiated using an initiator composed of 0.15 Wt % ethyl *p*-dimethylaminobenzoate (Acros, Pittsburgh, PA), 1.0 Wt % camphorquinone (Sigma Aldrich, St. Louis, MO), and 3.0 Wt % *p*-(octyloxyphenyl)-phenyliodonium hexafluoroantimonate (Gelest, Morrisville, PA). In preliminary studies, we found that photoinitiated silorane polymerization was not inhibited when filled with up to 50 Wt % of the ECHE-TMS surface modified glasses or up to 5 Wt % of the ECHE-TMS surface modified alumina nanorods.[20]

We used a commercially available mixture design-of-experiments software (Design Expert 7; Stat-Ease, Minneapolis, MN) to analyze the characteristics of the composite formulations tested (Table I). Replicates were added to the tested formulations to lower and balance the leverages of each data point. Each of these formulations was prepared by mixing the filler and the resin using a high-speed mixer (FlackTek, Landrum, SC) until visual inspection every 5 min confirmed complete mixing. The composite was allowed to rest 10–15 min after mixing, until visible air bubbles were removed and then used immediately to prevent premature polymerization. The properties of composite formulations 4 and 6 were not determined because they failed to polymerize.

Maximum exotherm temperature

Exotherm temperature was measured using a K-type thermocouple (Omega, Stamford, CT) affixed to a glass slide and slightly bent so that the tip of the thermocouple was positioned in the center of an acetal resin (Delrin®) washer (McMaster-Carr, Aurora, OH), which was also affixed to the glass slide with lab tape (Figure 2). Each composite formulation (0.6 g) was mounded to completely cover the tip of the thermocouple. The sample was then irradiated [12 mm diameter tip, 450 mW/cm^2 (Cure Rite, Dentsply Caulk, Milford, DE) at a distance of 3 mm] using a dental curing lamp (3M XL3000, St. Paul, MN) for 2 min. Specimens were inspected after testing, and results were excluded from further study if the tip contacted the glass slide or was not entirely covered with the composite. Temperature data were recorded using a data logger (OM-PLTC, Stamford, CT) at 1 Hz for 30 min postirradiation.

Flexural strength and modulus

Flexural specimens (25 mm $\times$ 2 mm $\times$ 2 mm) were formed in borosilicate glass tubes (VitroCom, Mountain Lakes, NJ) coated with silicone spray mold release (Mark V Laboratory, East Granby, CT) as per ISO specification 4049.[21] A pipette

TABLE I. Compositions of the Composites Formulated in Development of Material Property Estimation Method

Formulation number	Volume fraction			
	SM	DY5	M12	Nanorod
1	0.7312	0.0000	0.2688	0.0000
2	0.7754	0.1709	0.0390	0.0147
3	0.9848	0.0000	0.0000	0.0152
4	0.7108	0.2684	0.0000	0.0208
5	0.8733	0.1180	0.0000	0.0087
6	0.7108	0.2684	0.0000	0.0208
7	0.8863	0.0000	0.1049	0.0088
8	0.7201	0.1435	0.1258	0.0106
9	0.7352	0.0000	0.2433	0.0216
10	1.0000	0.0000	0.0000	0.0000
11	0.8355	0.0877	0.0768	0.0000
12	0.8355	0.0877	0.0768	0.0000
13	0.9164	0.0379	0.0332	0.0125
14	1.0000	0.0000	0.0000	0.0000
15	0.7815	0.1085	0.0951	0.0148
16	0.7877	0.0452	0.1521	0.0150
17	0.7044	0.2956	0.0000	0.0000
18	0.8863	0.0000	0.1049	0.0088
19	0.7044	0.2956	0.0000	0.0000
20	0.8578	0.0412	0.0874	0.0136
21	0.7312	0.0000	0.2688	0.0000
22	0.9848	0.0000	0.0000	0.0152
23	0.7201	0.1435	0.1258	0.0106
24	0.8733	0.1180	0.0000	0.0087
25	0.7352	0.0000	0.2433	0.0216
Additional composite formulation 1	0.7352	0.0000	0.2433	0.0216
Additional composite formulation 2	0.7886	0.0462	0.1485	0.0167
Additional composite formulation 3	0.7247	0.0839	0.1855	0.0059
Additional composite formulation 4	0.9154	0.0813	0.0000	0.0033

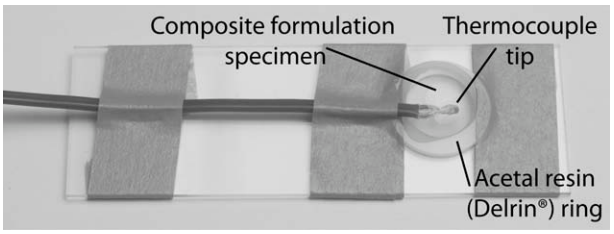

FIGURE 2. A photograph of the experimental setup for determining the maximum exotherm temperature.

was used to fill the molds with resin. The specimen was irradiated [12 mm diameter tip, 450 mW/cm^2 (Cure Rite, Dentsply Caulk, Milford, DE) at a distance of 3 mm] using a dental curing lamp (XL3000; 3M, St. Paul, MN) for 2 min along the top surface at three consecutive regions for 40 s each, 40 s in a scanning motion along the bottom of the glass mold, and then the specimen was removed from the glass. The method of photoinitiating specimens and induction of any overlapping regions have been shown to not have an effect on flexural properties.[22] The specimens were stored in phosphate-buffered saline (PBS), at 23 ± 1°C, for 24 h, after which, the specimen was loaded, until fracture, at a displacement rate of 3.7 mm/min in a four-point bend fixture with a support span of 20 mm on a BOSE mechanical tester (EnduraTEC ELF 3300, Eden Prairie, MN). Specimens with visible surface flaws, bubbles, or undistributed filler particles were excluded from the study. The resulting stress–strain curve was used to determine flexural strength (σ_B) and flexural modulus of elasticity (E_B).

Fracture toughness

The configuration of the specimens used in the fracture toughness tests was the same as that used in the flexural strength/modulus tests. Using steps detailed in ASTM D 1708-06a,[23] a 0.15-mm slotting cutter (Malco, Cranston, RI) was used to create a 0.6 mm deep notch on one face of the test specimen. The specimen was stored in PBS, at 23 ± 1°C, for 24 h after which it was loaded, on a materials testing machine (ELF 3300), at a displacement rate of 1.0 mm/min, until fracture. Specimens with visible surface flaws, bubbles, or undistributed filler particles were excluded from the study. The fracture toughness (K_{IC}) of the composite was determined as a function of the maximum load incurred before failure according to the standard.

Property estimation method

There were two steps in the method. In the first, for a given material property, the equation given below was fitted to the body of experimental results obtained from the 23 tested composite formulations. This equation relates the material property to the volume fractions of the fillers. In other words, the equation is of the form:

$$\begin{aligned} Y = a + b[\mathrm{SM}] + c[\mathrm{DY5}] + d[\mathrm{M12}] + e[\mathrm{NR}] + f[\mathrm{SM}][\mathrm{DY5}] \\ + g[\mathrm{SM}][\mathrm{M12}] + h[\mathrm{SM}][\mathrm{NR}] + i[\mathrm{DY5}][\mathrm{M12}] + j[\mathrm{DY5}][\mathrm{NR}] \\ + k[\mathrm{M12}][\mathrm{NR}] + l[\mathrm{SM}][\mathrm{DY5}][\mathrm{M12}] + m[\mathrm{SM}][\mathrm{DY5}][\mathrm{NR}] \\ + n[\mathrm{SM}][\mathrm{M12}][\mathrm{NR}] + o[\mathrm{DY5}][\mathrm{M12}][\mathrm{NR}] \end{aligned} \quad (1)$$

where [ ] denotes volume fraction of the filler or resin, and *a*–*o* are the corresponding coefficient estimates (Table II). The values of these coefficients, the standard error associated with each coefficient, and the coefficient of multiple determination for the equation were determined using a commercially available regression analysis software (Design-Expert 7.1.6, Stat-Ease, Minneapolis, MN).

In the second part, we synthesized four additional composite formulations whose compositional details were within the range of those used in the development of the estimation method (additional composite formulations 1–4 in Table I). For each of these additional formulations, T_{max}, σ_B, E_B, and K_{IC} were determined using the methods detailed above. In addition, for each of these additional formulations,

TABLE II. Equation Coefficients and Standard Errors for Model Output

	Maximum exotherm temperature		Flexural strength		Flexural modulus		Fracture toughness	
Component	Coefficient estimate	Standard error	Coefficient estimate	Standard error	Coefficient estimate	Standard error	Coefficient estimate	Standard error
A-SilMix	115.16	3.92	73.33	3.42	2.29	0.07	0.46	0.04
B-DY5	67.56	4.10	80.79	5.65	5.10	0.12	0.95	0.04
C-M12	92.87	5.56	104.86	26.11	3.92	0.54	0.74	0.06
D-Nanorods	6610.09	4630.80	−20672.71	70507.21	1324.03	1465.76	119.27	50.67
AB	27.60	21.91	−106.13	296.65	−8.01	6.17	−0.11	0.24
AC	37.91	26.29	−262.66	248.78	11.72	5.17	0.61	0.29
AD	−6703.78	4864.27	21175.63	74266.22	−1390.08	1543.91	−122.86	53.22
BC	49.72	29.79	103.19	414.16	13.96	8.61	1.06	0.33
BD	−7579.50	4783.65	21090.45	75838.89	−1549.26	1576.60	−127.25	52.34
CD	−7453.12	5005.54	21755.33	76913.36	−1391.98	1598.94	−127.10	54.77
ABC			814.65	2385.21	−36.34	49.59		
ABD			4231.67	6154.37	629.28	127.94		
ACD			6254.09	7321.42	−209.52	152.20		
BCD			−7323.69	8737.34	111.92	181.64		
R^2	0.9090		0.8111		0.9902		0.8474	

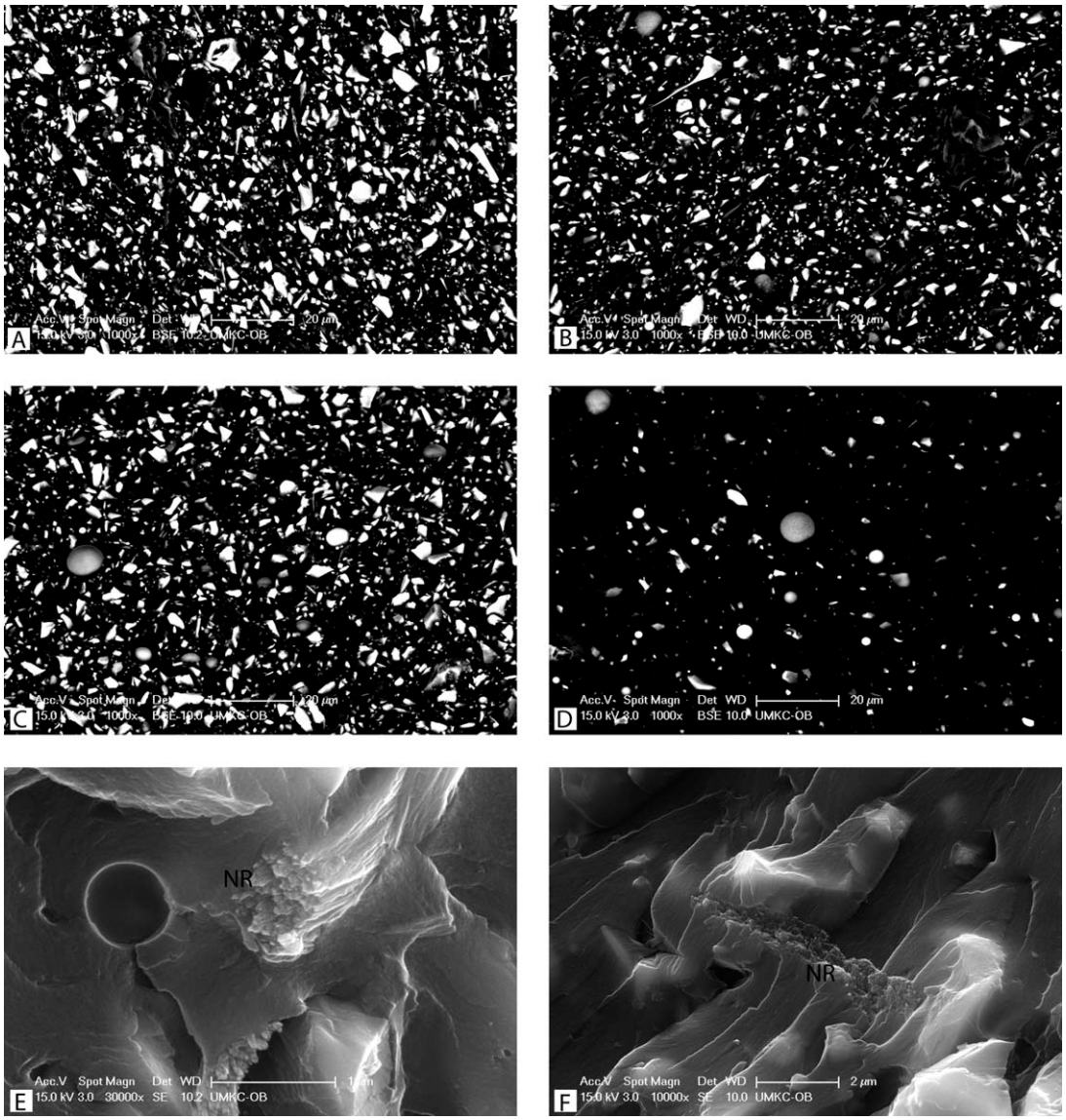

FIGURE 3. Morphologies of the fracture surface of flexural test specimens of additional composite formulations 1–4. Images (1000×) of additional composite formulations 1–4 (panels A–D) show uniform distribution of the glass filler particles (DY5 and M12). Higher magnifications of composite 1 (panel E, 30,000×) and composite 2 (panel F, 10,000×) reveal agglomerations of nanorod filler particles.

morphological details, in particular, the distribution of the filler particles within the matrix, were obtained using a scanning electron microscope (SEM; Field-Emission Environmental Scanning Electron Microscope FEI/Phillips XL30 ESEM-FEG, Phillips Electron Optics, FEI Company, Hillsboro, OR). SEM analysis of the fracture surfaces of flexural test specimens was used to verify if the filler particles were adequately distributed throughout the composite.

Statistical analysis

For each of the four additional composite formulations, a predicted material property obtained using Eq. (1) was compared with that obtained experimentally (T_{max}, $n = 3$; σ_B, $n = 8$; E_B, $n = 8$; and K_{IC}, $n = 8$) using a one-sample *t*-test (PASW Statistics 18, IBM Corporation, Somers, NY).

RESULTS

For each of the material properties determined, the fit of Eq. (1) to the experimentally obtained values for the 23 formulations was (1) good for formulations in which the filler was either of the two glass particles (Table II), which is attributed to good dispersal of these materials in the matrix (Figure 3); (2) poor for formulations in which the filler was alumina nanorods (Table II), which is attributed to agglomeration of these materials in the matrix (Figure 3); and (3) poor when combinations of the volume fractions of the fillers in the composites were considered (Table II). The property estimation method was considered validated (Table III).

DISCUSSION

We have identified a biocompatible silorane resin with potential applications in many health fields, such as

TABLE III. Predicted and Observed Results of Additional Composite Formulations

	Maximum exotherm temperature (°C)		Flexural strength (MPa)		Flexural modulus (GPa)		Fracture toughness (MPa $m^{1/2}$)	
	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
Composite 1	74.0 (4.0)[a]	76.6 (68.2–85.1)	59.9 (4.6)	72.0 (64.3–79.7)	4.96 (0.09)	5.45 (5.29–5.61)	0.86 (0.07)[a]	0.84 (0.75–0.93)
Composite 2	83.7 (1.5)	88.4 (83.1–93.6)	66.5 (5.6)	77.0 (27.4–126.6)	4.26 (0.07)	4.58 (3.55–5.61)	0.83 (0.06)[a]	0.84 (0.78–0.90)
Composite 3	77.3 (2.1)	83.7 (73.1–94.3)	61.5 (2.5)	98.5 (41.0–155.9)	4.66 (0.08)	5.13 (3.94–6.33)	0.86 (0.03)[a]	0.87 (0.76–0.99)
Composite 4	110.7 (10.1)[a]	103.5 (94.9–112.2)	60.0 (3.9)	65.6 (15.7–115.6)	2.73 (0.04)	1.78 (0.74–2.82)	0.59 (0.05)	0.53 (0.44–0.63)

For the observed data, numbers in parentheses are standard deviation.
For the predicted values, numbers in parentheses are 95% confidence intervals.
[a] $p > 0.05$ between predicted and observed measurements.

orthopaedics. For instance, the silorane could provide an alternative fracture stabilization technique for comminuted fractures. Previous studies have shown the silorane is biocompatible and has good mechanical properties. However, the effect of various fillers on the maximum exotherm temperature, flexural strength, flexural modulus, and fracture toughness of silorane composites has not been determined. The goal of this article was to develop a method for estimating these properties in the silorane composite.

Although their functions are different, the desirable properties of the silorane composite have been adapted from values given in acrylic bone cement standards. The ISO standard for acrylic bone cement[24] requires a maximum exotherm of less than 90°C, a flexural strength greater than 50 MPa, and a flexural modulus greater than 1.8 GPa. Fracture toughness is also important to predict increased fracture resistance. Fracture toughness ranges depending on test method from 1.0–2.3 MPa $m^{1/2}$ for commercially available acrylic bone cements.[25–27] However, all of these measurements are greatly dependent on test method. The results of this study indicate that the silorane composites containing a combination of fillers (DY5, M12, and/or nanorods) met the suggested bone stabilizer requirements with exotherms as low as 65°C, flexural strength up to 63.8 MPa, and flexural moduli up to 5.16 GPa. However, the fracture toughness of photoinitiated silorane composites (0.42–0.96 MPa $m^{1/2}$) was lower than the range for commercially available acrylic bone cements. One solution identified by the developed method that meets the ISO 5833[24] criteria contains 85.8% silorane, 13.1% DY5, 0.0% M12, and 1.1% nanorods. The predicted properties of this formulation are an exotherm of 88.1°C, flexural strength of 74.0 MPa, and a flexural modulus of 3.7 GPa. However, it must be noted that the properties calculated in this study were conducted on smaller specimens to accommodate available material size. To fully understand the ability of this novel material to meet bone cement standards, tests must be conducted on the optimized material using standard methods.

The flexural properties, in particular flexural strength, were the least accurately predicted from the model. Although a similar trend was seen in the predicted versus observed modulus values, the range of observed strengths for the four additional composites was small (59–66 MPa), and the values were lower than the predicted values (72–99 MPa). This suggests premature failure of the test specimens, although specimens with visible flaws (voids, filler agglomerations, etc.) were excluded from the study. Further analysis, such as high-magnification observation of fracture surfaces, was not utilized to identify flawed specimens, but may have excluded additional samples from the study, resulting in greater mechanical property values. The flexural strength of the additional composite that did not contain nanorods (composite 4) was the closest to fitting its predicted value. Furthermore, the standard error of many of the composites containing nanorods (denoted by D in Table II) was greater than the estimated coefficient. These results may be due to incomplete mixing of the nanorods in the composite, which showed some agglomeration in SEM analysis (Figure 3).

Inhomogeneity of filler distribution may lead to stress concentrations. These stress concentrations would not greatly affect the global properties, such as modulus or exotherm. However, they could cause premature failure, thus lowering the fracture strength of the resulting composite.

It is known that mixture design-of-experiments method suffers from combinatorial explosion when dealing with multi-component mixtures.[28,29] Combinatorial explosion describes the inability to compute the outcome due to the intractability of the number of inputs; in other words, too many inputs (components) gives far too many outcomes (possible behaviors) to compute. Solvason et al.[29] further described how the visualization of multiple components in the design space is problematic. Given a response change over the design space, it is difficult to determine which one component (or combination) causes that response, or how much each component affects the overall response. Internal points within the design space cannot be predicted using the response coefficients if we cannot discern the sizes of the coefficients accurately. Check points, such as those used in this study, help to discern the accuracy of the model for prediction. More points in general also assist and can be chosen to be more orthogonal with respect to the other components. To develop the predictive equations in this study, we used material property values obtained from 23 composite formulations. The number of formulations was determined through analysis of the leverage of each formulation on the resulting model. Overall, leverages were decreased by adding replicates to below 0.5, with a homogenous range from 0.22 to 0.48. Due to combinatorial explosion, however, we cannot assess all behaviors using the model. Therefore, the solution given in this study is simply a predictive tool.

Further limitations of regression analysis include the fact that the solution may not fit the data and/or the detail of information between model points may not be fitted well (if there is a strong nonlinear response component). In this study, however, we do not expect a strong, nonlinear response. Also, the fitting was assessed using the additional composite formulations to determine fitting error (as opposed to experimental errors which are assessed by repeated testing at constant conditions). A way to further test the fitting, though, would be to transform the data using a suitable function to 'flatten' the response, fit to a model, and then transform the solution for comparison. If different functions are used, however, it can be difficult to compare them mathematically; thus, this process may be more of an academic exercise than being useful for validating a particular model.

Overall, based on the fact that the majority of standard errors for the coefficient estimates were reasonably low, and the observed properties for the additional composites were within the predicted ranges, the method of prediction developed in this study appears to be valid for determining maximum exotherm temperature, flexural strength, and flexural modulus based on the additional composites. In contrast, the standard errors of the estimates of the coefficients for properties of composites that contained nanorods (D, AD, BD, etc.) were quite large; thus, caution should be taken when predicting properties of nanorod-containing composites.

As with any study, there are limitations. As mentioned previously, the flexural strength results may indicate incomplete filler dispersion. Future studies will incorporate advanced mixing techniques, such as ultrasound, and verify fracture site filler particle homogeneity with SEM analysis to improve future models. Furthermore, a more accurate model of the physiological environment, such as placing specimens in contact with bone, could be used to provide more meaningful exotherm measurements. This study was conducted on photoinitiated materials because this is the method currently used in the commercial silorane product, and we wanted to limit the number of variables. Although this study focused solely on photoinitiated materials, the ideal silorane composite for orthopedic use will be chemically initiated. Photoinitiation requires an external light source which is not ideal for the majority of orthopedic applications. The chemically initiated silorane will likely have a slower polymerization speed, so it is likely that as the silorane composite formulation is optimized the peak exotherm will also be decreased.

CONCLUSIONS

The objective of this study was met through the development of a method of predicting the mechanical and maximum exotherm temperature properties of the photoinitiated silorane composite. The silorane composite investigated in this study met the suggested material properties (exotherm, flexural strength, flexural modulus, and fracture toughness) for a bone stabilizer. For some of the composite formulations that contain alumina nanorods, the predicted values are up to 60% greater than observed flexural strength values, and flexural modulus predicted values are 10% greater than those observed. It is suggested that this is due to poor dispersal of the nanorods in the matrix. Improved filler distribution may be able to improve model accuracy.

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Silorane resin supports proliferation, differentiation, and mineralization of MLO-A5 bone cells *in vitro* and bone formation *in vivo*

J. David Eick,[1] Cielo Barragan-Adjemian,[1] Jennifer Rosser,[1] Jennifer R. Melander,[1] Vladimir Dusevich,[1] Rachel A. Weiler,[2] Bradley D. Miller,[2] Kathleen V. Kilway,[2] Mark R. Dallas,[1] Lianxing Bi,[1] Elisabet L. Nalvarte,[1] Lynda F. Bonewald[1]

[1]Department of Oral Biology, School of Dentistry, University of Missouri—Kansas City, Kansas, Missouri 64108-2784
[2]Department of Chemistry, University of Missouri—Kansas City, Kansas, Missouri 64108-2784

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Abstract: Methyl methacrylate used in bone cements has drawbacks of toxicity, high exotherm, and considerable shrinkage. A new resin, based on silorane/oxirane chemistry, has been shown to have little toxicity, low exotherm, and low shrinkage. We hypothesized that silorane-based resins may also be useful as components of bone cements as well as other bone applications and began testing on bone cell function *in vitro* and *in vivo*. MLO-A5, late osteoblast cells, were exposed to polymerized silorane (SilMix) resin (and a standard polymerized bisGMA/TEGDMA methacrylate (BT) resin and compared to culture wells without resins as control. A significant cytotoxic effect was observed with the BT resin resulting in no cell growth, whereas in contrast, SilMix resin had no toxic effects on MLO-A5 cell proliferation, differentiation, nor mineralization. The cells cultured with SilMix produced increasing amounts of alkaline phosphatase (1.8-fold) compared to control cultures. Compared to control cultures, an actual enhancement of mineralization was observed in the silorane resin-containing cultures at days 10 and 11 as determined by von Kossa (1.8–2.0 fold increase) and Alizarin red staining (1.8-fold increase). A normal bone calcium/phosphate atomic ratio was observed by elemental analysis along with normal collagen formation. When used *in vivo* to stabilize osteotomies, no inflammatory response was observed, and the bone continued to heal. In conclusion, the silorane resin, SilMix, was shown to not only be non cytototoxic, but actually supported bone cell function. Therefore, this resin has significant potential for the development of a nontoxic bone cement or bone stabilizer. © 2012 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 100B: 850–861, 2012.

Key Words: siloranes, bone stabilization, mineralization, MLO-A5 cell line, osteotomy

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INTRODUCTION

Bone cements have been used for decades in the fixation of prosthetic devices. Polymethyl methacrylate (PMMA)-based cement is a well-recognized conventional bone cement that provides reasonably good clinical results; however, severe problems are still associated with its use, such as, cytotoxicity, thermal injury, respiratory and cardiovascular complications in addition to polymerization shrinkage, which can affect the stability of the implant.[1–5] The interaction between resin and bone causes internal stress that can lead to gap formation between the PMMA and the bone.[6]

Silorane-based resins have been developed by 3M-ESPE[7] for the production of dental composite materials. These resins have proved to have superior characteristics to bisGMA/TEGDMA (bisphenol A glycidyl methacrylate and triethylene glycol dimethacrylate), the two usual monomer components of dental composites. The term "silorane" was introduced to represent hybrid monomer systems that contain both siloxane and oxirane structural moieties. Siloranes contain a cyclosiloxane backbone, which imparts hydrophobicity[8]; in addition, they contain cycloaliphatic oxirane sites that have high reactivity and present less shrinkage during polymerization than methacrylates.[9,10] Some cyclosiloxanes have been reported to undergo cationic ring-opening polymerization with volume expansion.[11] These resins exhibit excellent biocompatibility. Cytotoxicity ratings are as good as or better than those for typical methacrylate dental monomers, such as bisGMA-based polymer. They also are nonmutagenic.[12–14] Marginal integrity and microleakage of silorane-based restorative systems are reported to be superior to methacrylate-based systems.[15] Shear bond strength and other mechanical properties have also been studied and

Correspondence to: L. F. Bonewald; e-mail: bonewaldl@umkc.edu
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found to be better than the methacrylate resins.[16–20] It was also shown that a silorane-based dental composite can effectively bond to bone.[21]

In order to begin to develop better bone cements, we analyzed the effect of silorane-based resins on bone cell function *in vitro* and *in vivo*. One aim of the present study was to analyze the effect of silorane-based resin on bone cell proliferation, differentiation, and mineralization. MLO-A5 cells were used as an *in-vitro* model for bone formation. MLO-A5 cells are a post-osteoblast/pre-osteocyte-like cell line established from the long bones of 14-day-old mice expressing the large T-antigen driven by the osteocalcin promoter.[22] These cells express extremely high levels of alkaline phosphatase and osteocalcin, as well as, osteopontin, periostin, bone sialoprotein, and PTH type 1 receptor compared to primary osteoblasts and osteoblast cell lines.[22] Previously, we had shown that the MLO-A5 cells mineralize in culture, forming sheets not nodules, and that this mineralized matrix contains a ratio of calcium to phosphorus similar to bone.[23] These cells will mineralize in the absence of beta glycerolphosphate (βGP) in 6–7 days, but this process is accelerated by the addition of an external source of phosphate. Spectra obtained by Fourier transform infrared spectroscopy, FTIR, of these cultures were shown to be very similar to normal bone.[22,23] The MLO-A5 cells appear to be a good model for *in vitro* lamellar bone formation. These cells were used for the present study in order to obtain insight into the potential mechanisms by which bone would form in the presence of silorane-based resins in comparison to the effect of a methacrylate composite, bisGMA/TEGDMA (BT). A second aim of the study was to examine the effects of silorane resin on bone cell function *in vivo* and to determine if the resin elicited an inflammatory response. We chose to use the standard femoral osteotomy approach in mice. The silorane resin was used to stabilize the osteotomy was up to one month for radiographic and histological analysis. Overall, silorane resin had little or no negative effects on bone cell function.

MATERIALS AND METHODS

Preparation of resins

The resins were prepared as described previously.[17,24,25] Briefly, the silorane-based resin SilMix, is a 1/1 wt/wt mixture of two silicon-containing oxiranes, bis[2-(3{7oxabicyclo[4.1.0]heptyl})-ethyl]methylphenyl silane (PHEPSI)[26] and 2,4,6,8-tetrakis(2-(7-oxabicyclo[4.1.0]heptan-3-yl)ethyl)-2,4,6,8-tetramethyl-1,3,5,7,2,4,6,8-tetraoxatetrasilocane (CYGEP)[27] (see Figure 1). A conventional methacrylate-based matrix resin bisGMA (BisGMA/TEDGMA 50/50) used in dental composites was used as a control. For the drop method, the silorane (SilMix) and methacrylate (Z250: bisGMA/ TEGDMA) resins were obtained from 3M-ESPE (St. Paul, MN, and Seefeld, Germany). For the rest of the samples, the methacrylate monomer system (BT) was a 1:1 mixture by weight of two methacrylates, bisGMA (purity: 93%, Esstech, Inc.) and TEGDMA (purity: 97%, Sartomer). With the exception of the resin drop samples, the silorane monomers (SilMix) were synthesized using an adapted procedure for PHEPSI[26] and CYGEP[27] resulting in a >95.8% purity as determined by ^{1}H NMR spectroscopy. All resin samples were prepared at room temperature (~ 20°C) and under yellow light in order to prevent premature polymerization. The photoinitiator system (see Figure 1) used for all the resins consisted of phenyl[*p*-2-hydroxytetradecyloxyphenyl]iodonium hexafluoroantimonate (PI, Gelest, Inc., Tullytown, PA); camphorquinone (CQ, Aldrich, Milwaukee, WI), and ethyl-4-dimethylaminobenzoate (EDMAB, Fisher Scientific / ACROS, Pittsburgh, PA). The photoinitiator and monomer systems were combined using a speed mixer and mixed for periods of 5–15 minutes depending on the amount of material. The final mass composition was 0.15% EDMAB, 1.0% CQ, 3.0% PI, and 95.85% SilMix and the BT composition was 0.15% EDMAB, 1.0% CQ, 3.0% PI, and 95.85% BisGMA/TEGDMA. Resins were prepared the same day and used within a 2-h period after preparation.

Resin polymer characterization

To ensure that resin polymerization was complete, the degree of conversion (DC) of the SilMix and BT resins was analyzed using FTIR spectroscopy (Perkin–Elmer Spectrum One, ATR sampling mode) analysis. A Delrin mold was fixed on the FTIR instrument; the resin (80 mg) was added to the mold, and the resin polymerized via light cure with a 3M curing lamp, (3M XL3000, St. Paul, MN, 450 mW/cm^2 light intensity) for three 40-sec intervals. The solid discs ($n = 6$) were detached from the mold, and half were subjected to 2 h sterilization below laminar hood UV light (1 h per side). The other half were allowed to dark cure. FTIR spectra were collected from the unpolymerized resin at 2 min after light cure and at 4 h after light cure (polymer with dark cure) ($n = 3$). The FTIR spectra were baseline corrected, and the DC was calculated for each polymer sample using a polymerization dependent peak [BT: 1638 cm^{-1} (C=C), and SilMix: 883 cm^{-1} (oxirane ring opening)], which was compared to an internal standard [BT-1608 cm^{-1}(phenyl), and SilMix 1258 cm^{-1}(C—O in ring)]. The DC was calculated as the difference in the peak ratios from the unpolymerized resin assuming that the unpolymerized resin spectra represented no (0%) polymerization.

Culture of MLO-A5 cells with resin

Approximately 50 μL of the resin was dropped into the center of a NUNC brand Thermanox coverslip (Electron Microscopy Science Hatfield, PA) and polymerized with a 3M curing lamp (450 mW/cm^2 light intensity) for three 40-sec intervals. Thermanox coverslips with polymerized resin drops of 5-mm diameter or without resin for control were used in triplicate. The coverslips were placed in 24-well plates, and MLO-A5 cells were plated at a density of 3.5×10^4 cells/cm^2 in α-MEM containing 5% fetal bovine serum (FBS) and 5% calf serum (CS). Cells were cultured for 24, 48, and 72 h, then washed with PBS, and harvested with trypsin-EDTA. The cell number was measured using a Coulter Counter (Z1 Coulter particle counter, Beckman Coulter Fullerton, CA). In these experiments, it was observed that cells did not attach well to the resin drop

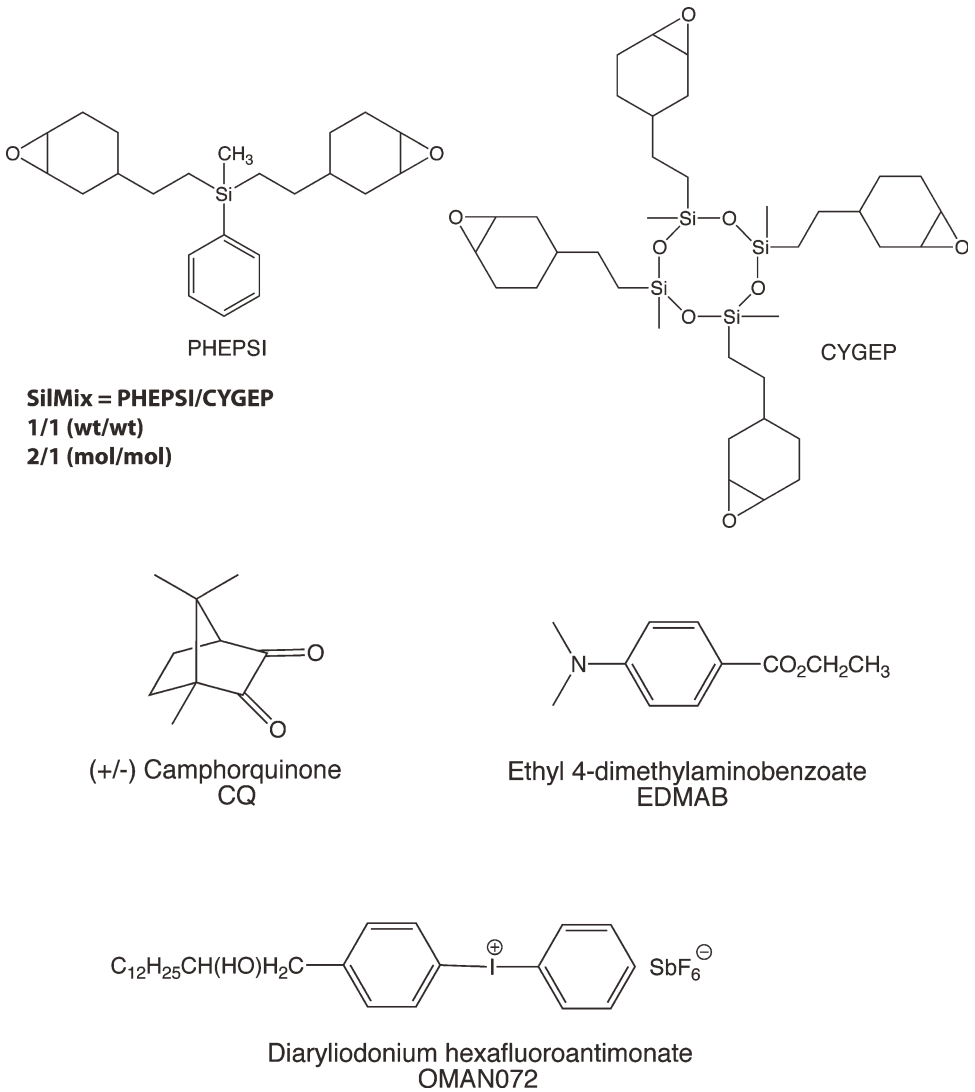

FIGURE 1. Chemical structure of siloranes and photoinitiator system used for the resins. The silorane resin used for the *in vitro* bone cell assays and *in vivo* is composed of SilMix a 1/1 wt/wt ofPHEPSI/CYGEP.

surfaces; therefore a second experimental design generated discs of SilMix and BT polymer of 9 mm diameter by 0.7 mm thickness. Discs were prepared by placing 80 mg of the freshly mixed resin into Delrin ring molds (McMaster-Carr, Elmhurst, IL), which were fixed on glass slides. The resin was light cured (3M curing lamp, 450 mW/cm^2 light intensity) for three 40-sec intervals at a distance of 1 mm from the top of the sample. Solid polymer discs were detached from the molds and sterilized under laminar hood UV light for 1 h on each side. The discs, which covered the entire bottom surface of 48-well culture plates, were placed in the wells prior to addition of MLO-A5 cells at a density of 3.5×10^4 cells/cm^2. After 24 and 48 h of incubation, cell attachment and proliferation were assessed by measuring the cell number using the Coulter counter assay and the Trypan blue dye exclusion (TBE) assay.

The cell monolayer was washed with 0.5 mL of PBS, and then pooled with the respective supernatants. Trypsin/EDTA (0.2 mL) was added to the cell layer and incubated for 2–3 min at 37°C/5% CO_2. Meanwhile, the cells in the supernatants were pelleted and treated with 0.05 mL Trypsin/EDTA at 37°C/5% CO_2. Trypsinized cell suspensions were pooled (1.25 mL), then centrifuged for 2 min (5000 rpm). The obtained cell pellet was re-suspended in 100 μL of PBS. In each microcentrifuge tube, 20 μL of 0.4% trypan blue dye was added, mixed thoroughly, and allowed to stand for 3–5 min at room temperature. A hemacytometer was loaded with 10 μL cell suspension, and cells were counted under a microscope. Similar procedures were performed with cells grown on the polystyrene control wells.

Cell viability in response to polymer extracts

To study the effect of leachables on cell viability, sterilized discs were inserted into 48-well plates and washed with 0.5 mL of culture media for 1 h at 37°C/5% CO_2. The used media was discarded, and fresh media (0.5 mL) was added to the polymer discs as well as the control wells ($n = 4$) and incubated for 48 h at 37°C/5% CO_2. In parallel and in

the same plate, the 1 h pre-incubated wells were seeded with 0.5 mL of 3.5×10^4 cells/mL and grown for 48 h. Then, the media in these wells containing cells were removed and replaced with 0.5 mL of conditioned media exposed to the discs (assumed to contain leachables from the polymer discs). After incubation for 24 h at 37°C/5% CO_2, cell viability was measured using the MTT assay. For the MTT assay, 50 μL of 5 mg/mL of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma M5655] in phosphate buffered saline (PBS) were added to the culture plates and returned to the incubator. After 4 h of incubation time, the supernatants with unreacted MTT were discarded, and the purple formazan crystals in the cells were dissolved by adding dimethyl sulfoxide (DMSO, 0.5 mL). Formazan/DMSO aliquots were read at 550 nm in a 96-well plate reader (Molecular Devices Corp., Menlo Park, CA).

Culture of cells for mineralization. Due to low attachment of cells on resin surfaces, mineralizaton analysis was carried out using resin drops. Approximately the resin (50 μL) was dropped into the center of a NUNC brand Thermanox coverslip (Electron Microscopy Science Hatfield, PA) and polymerized with a 3M curing lamp, (450 mW/cm^2 light intensity) for three 40-sec intervals. To rule out any effects of cells potentially settling in the middle of the well and being displaced by the resin for successive experiments, resin (20–30 μL) was placed either in the center of the coverslip or toward the side, but not touching the edge of the coverslip. After polymerization, the sample and Thermanox discs were washed with PBS and placed in 24-well plates for sterilization under laminar hood ultraviolet light for ~ 1–2 h, before use.

Thermanox coverslips with polymerized resin drops or without resin for control were used in triplicate. The coverslips were placed in 24-well plates, and MLO-A5 cells were cultured as described previously.[22] MLO-A5 cells were plated at a density of 3.5×10^4 cells/cm^2 in α-MEM containing 5% fetal bovine serum (FBS) and 5% calf serum (CS). Upon confluency, designated day 0, media was removed, and the cells were incubated in mineralizing media, α-MEM with 10% FBS, 4 m*M* of β-glycerolphosphate, βGP, and 100 μg/mL of ascorbic acid. Media were changed every two days through 11 days.[23]

Alkaline phosphatase assay

MLO-A5 cells were cultured on cover slips for 6 days under mineralization conditions as described above and analyzed for alkaline phosphatase enzyme activity. Briefly, cells were fixed with 10% buffered formalin for 10 min and washed with PBS two times. Fresh solution containing 0.033% NBT (nitro blue tetrazolium) and 0.017% BCIP (5-bromo-4-chloro-3-indolyl phosphate) in ALP buffer (100 m*M* sodium chloride, 5 m*M* magnesium chloride, 100 m*M* Tris-HCl, pH 9.5) was added to the cultures and incubated at 37°C for 20 min. The purple stained area was measured using a semiautomated imaging system as described previously.[22,23]

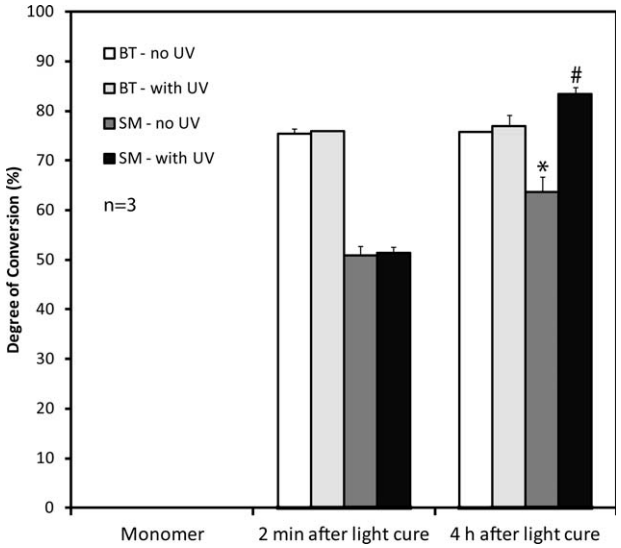

FIGURE 2. Overall degree of conversion for bisGMA/TEGDMA (BT) without ultraviolet light, UV (open bars) or with UV sterilization treatment (dotted bars) and SilMix (SM) polymers without UV (hatched bars) and with UV (gray bars). The DC of 4 h after light cure and UV sterilized polymers are representative of the DC of discs used for the cell proliferation tests ($n = 3$). *Significant change ($p < 0.05$) in the DC of the SM without and with UV light treatment at 4 h, as well as the DC of the SM from 2 min to 4 h. #Significant increase ($p < 0.05$) in the DC of SM-with UV relative to BT-with UV using three-way ANOVA.

Immunohistochemical staining for collagen type 1

MLO-A5 cells were plated on coverslips and cultured as described above. After 6 days in culture, the cells were washed with PBS (two times), then fixed with 95% ethanol for 5 min and washed with PBS (three times). The cultures were then incubated with blocking solution, (PBS + 1% horse serum + 0.05% NaN_3) for 2 h at room temperature, followed by incubation with polyclonal antibody to type I collagen, LF-67, that recognizes the C-telopeptide of collagen type 1 (the antibody was kindly provided by Dr. Larry W. Fisher, National Institutes of Health, Bethesda, MD, USA). A 1:400 dilution in PBS + horse serum was added for 1 h at room temperature, followed by incubation with Cy-3 conjugated donkey anti-rabbit IgG in blocking solution, 1:250 for 1 h and followed by washing with PBS (six times). The cells were then examined, and photos were taken using fluorescence microscopy (Nikon eclipse E800 microscope).[23]

Scanning electron microscopy (SEM)

MLO-A5 cells were cultured on coverslips for 24 and 48 h as well as 6 and 10 days. At the end of the culture, the cells were gently washed with PBS, and fixed with 10% formalin for 20 min, washed again with PBS and dehydrated in a graded series of ethanol, and dried using hexamethyl disilazone (HMDS) for 5 min. After dehydration, the coverslips were attached to SEM stubs and sputter-coated with gold-palladium. The gold-palladium-coated cultures were examined using a FEI/Philips XL30 field emission environmental scanning electron microscope. An accelerated voltage in a range of 15 to 25 KeV was used for the secondary and

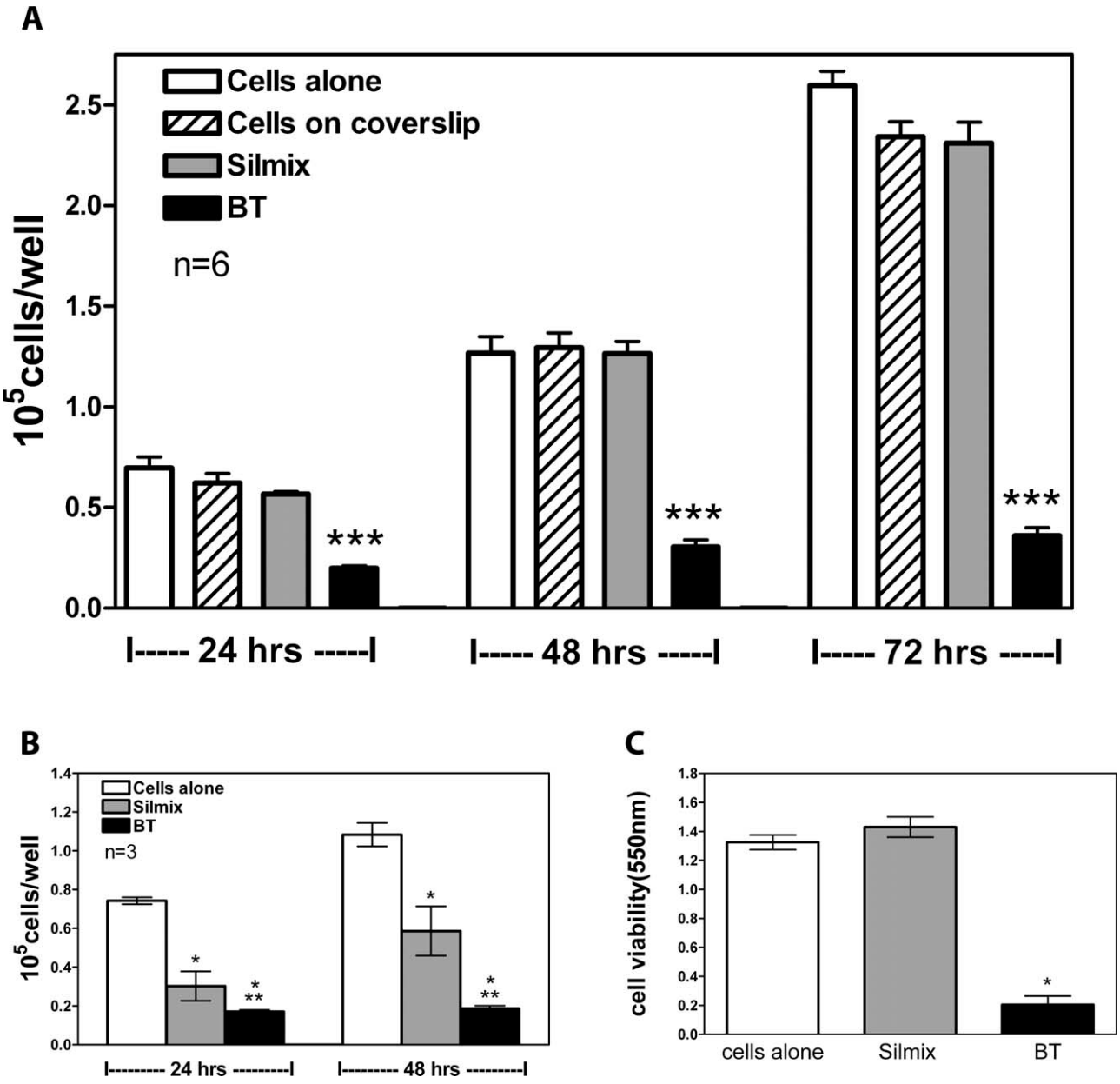

FIGURE 3. Effects of silorane and BT resins and resin leachables on MLO-A5 cell proliferation. Effects on cell number using resin drops (A) and resin discs (B). Silorane and BT disc media conditioned for 48 h added to MLO-A5 cells (C). ***Significantly different from SilMix ($p < 0.001$); *significantly different from cells alone ($p < 0.05$; $n = 3$); **significantly different from SilMix ($p < 0.05$) using one-way ANOVA and Tukey post test ($n = 3$).

backscatter electron imaging. For X-ray microanalysis EDS, the cultures were carbon-coated and examined with 15 KeV accelerating voltage. X-ray spectra and maps for calcium and phosphorus distribution were acquired.[23]

Von Kossa staining for phosphate quantification

The MLO-A5 cultured cells were washed with PBS and fixed with 10% buffered formalin for 10 min The samples were washed with water several times before a 2% silver nitrate solution was added and the plates exposed to UV light for 20 min and followed by rinsing with water. Five percent sodium thiosulfate was added for 3 min before rinsing. A modified van Gieson stain was then used as a counterstain following the von Kossa stain. This stain consisted of five parts 1% acid fuchsin and 95 parts picric acid, which was added for 5 min followed by washing with 95% ETOH (two times), 100% ETOH (two times), and then air drying before analysis. The mineralized area and total area were measured using a semiautomated imaging system as described previously.[22,23] Briefly, the area of von Kossa-stained matrix was quantified by automated image analysis using a video analysis program (Jandel Scientific, San Rafael, CA) linked to a video screen camera (CCD/RGB; Sony Corp., Park Ridge, NJ) and microscope (model BH2; Olympus Corp., Precision Instruments Division, Lake Success, NY) equipped with metallurgical lenses.

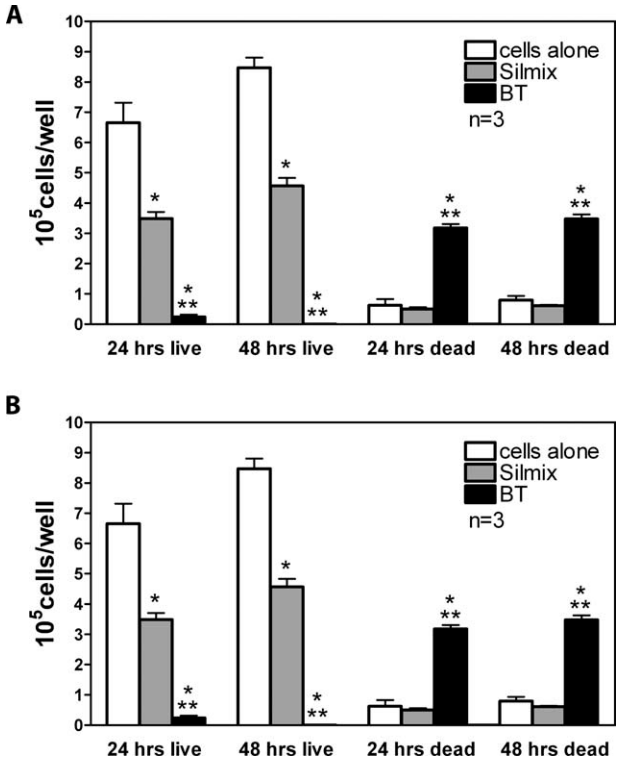

FIGURE 4. Effects of silorane and bisGMA/TEGDMA (BT) resins on MLO-A5 cell proliferation and attachment to polymer disc surfaces. (A) Effects of SilMix and BT on live and dead cell number. (B) In comparison to BT, most of the cells in wells with SilMix were viable with a percentage of live cells greater than 87% and similar to the controls. Compared to the respective time, *significantly different from cells alone ($p < 0.05$); **significantly different from SilMix ($p < 0.05$) using one-way ANOVA and Tukey post test.

Alizarin red staining for calcium quantification

The MLO-A5 cells were cultured for mineralization and fixed in formalin as mentioned previously above. Fixed cultures were washed three times with TBS (Tris-buffered saline) and then stained with 4 n*M* alizarin red S dye (AR-S) for 5 min. Cultures were then rinsed with water followed by a 15-min wash with TBS to reduce nonspecific AR-S stain. The mineralized areas were measured using a semiautomated imaging system as described previously.[23]

Stabilization of osteotomized murine femuri with SilMix

Surgical procedures. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of authors' institution. Eight 12-week old C57black6 mice were housed in the animal care facility under a 12-h/12-h light/dark cycle. The mice were anesthetized with 3.5% isoflurane and maintained with ketamine/dexdormitor (75/0.25 mg/kg body weight, intraperitoneally). The skin over the right hind limb was shaved, swabbed with betadine, and draped under aseptic conditions. Using the sterile instruments, a 1.5-cm skin incision was made on lateral aspect of thigh extending from the vastus lateralis muscle to the patellar ligament insertion, preserving the patellar ligament. The patella was retracted medially with the knee extended. The knee was slowly flexed to expose the intercondylar notch. The intramuscular septum between the vastus lateralis and hamstring muscles was separated using blunt dissection, and the periosteum was incised to expose the femur. A transverse fracture of the femur was created using an electrical round saw. A 0.7-mm K-wire was gently inserted into the intercondylar entry point, through the fractured femur, to the appropriate depth (approximately the level of lesser trochanter), which served as intramedullary fixation for the fracture to prevent angulations or displacement. SilMix resin (50–70 μl) was applied around the fracture site and cured using a dental curing lamp for 20 sec (three times. After polymerization of SilMix resin, the stability of the fixed femur was evaluated, then the K-wire was removed. The capsule and skin were sutured with 4–0 nylon. The animal was allowed to fully recover in a separate cage on a warming pad and was allowed activity *ad libitum*. An analgesic (buprenorphine hydrochloride, 0.05

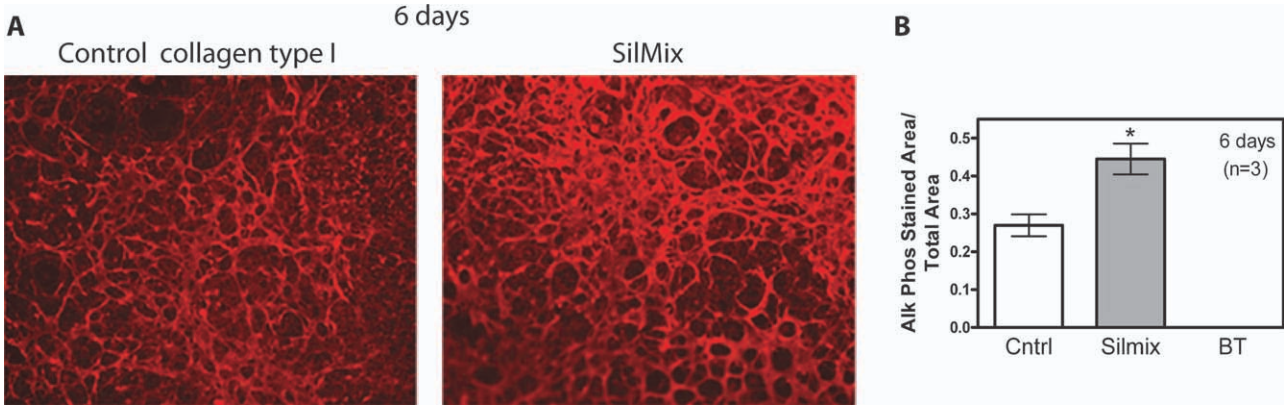

FIGURE 5. Effects of silorane on collagen matrix formation and alkaline phosphatase. Immunohistochemical staining of collagen type 1 fibers in the control (left) and SilMix (right) revealed an intact collagen network that appeared thicker in the SilMix resin drop cultures (A). (×10 magnification). No negative effects were observed on collagen matrix formation at 6 days of culture. At 6 days, significantly elevated alkaline phosphatase was observed in the SilMix containing cultures compared to the control. No alkaline phosphatase was detected in the BT cultures at 6 days (B). *Significantly different from cells alone ($p < 0.05$) and from BT ($p < 0.001$) using one-way ANOVA and Tukey post test. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

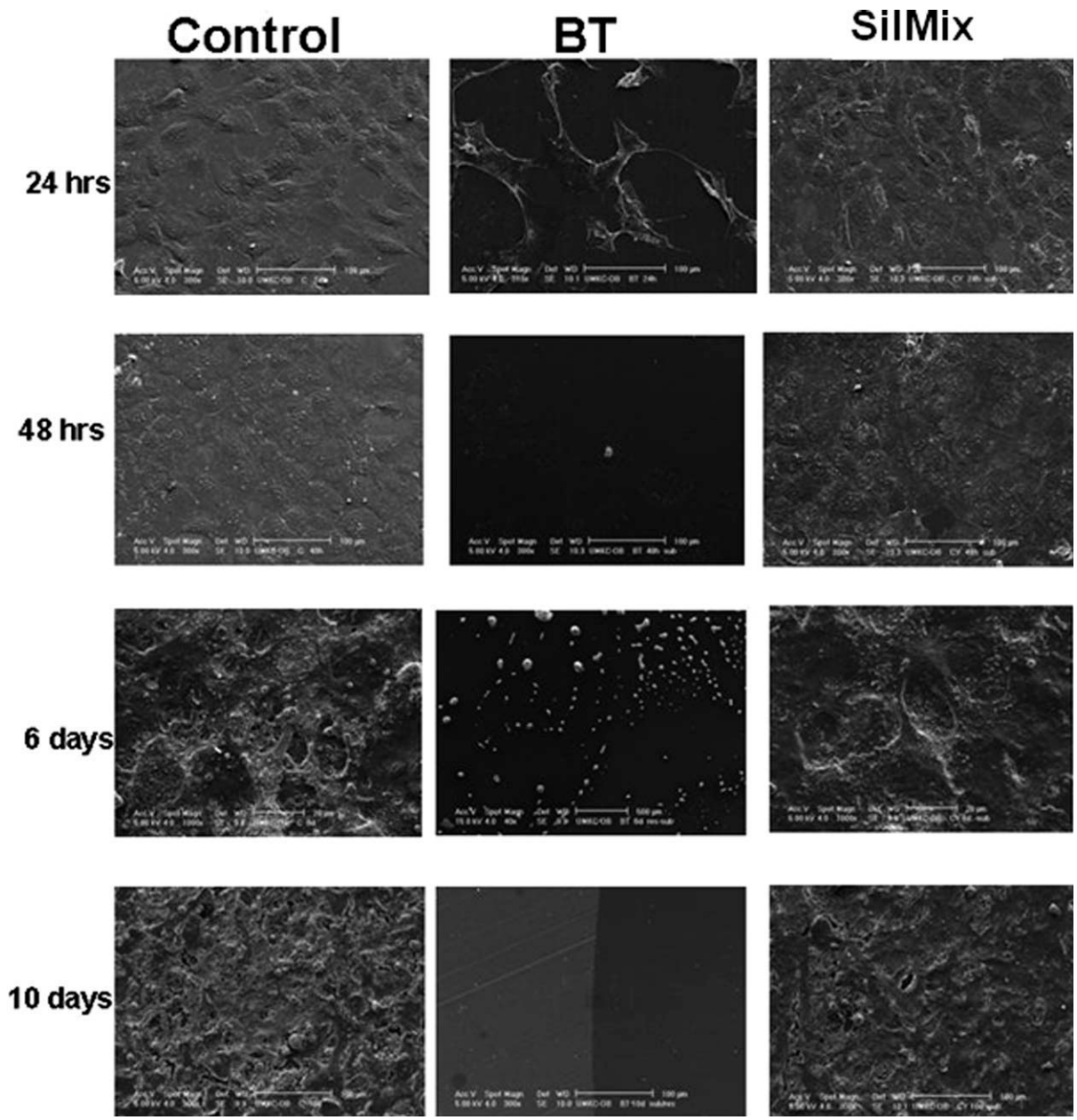

FIGURE 6. Secondary electron micrographs of MLO-A5 cells cultured for 24 and 48 h, and 6 and 10 days on cover slips with resin drops. At 24 h, the morphology of cells exposed to the SilMix appears normal, but with some membrane ruffling. The BT exposed cells appear necrotic. By 48 h, no cells are visible in the BT cultures. In contrast, the SilMix cultures show normal cell growth and matrix formation and appear healthy at 10 days of culture (scale bars = 100 μm except for 6 days at 20 μm).

mg/kg) was administered subcutaneously twice per day during three postoperative days.

Radiographic evaluation. At days 0, 7, 14, 21, and 28 postsurgery, the animals were anesthetized with ketamine/dexdormitor (75/0.25 mg/kg body weight, intraperitoneally). Radiographs of the femora were obtained using a Faxitron MX-20 (Faxitron X-Ray LLC, Lincolnshire, IL) at 26 KV and 10 sec. The fracture healing, angulation, or displacement of SilMixresin stabilized osteotomized femora was evaluated.

Histological assessment. The animals were sacrificed at days 7 ($n = 4$) or 28 ($n = 4$) postsurgery. The SilMix resin stabilized femora with surrounding tissues were harvested, fixed in 10% buffered formaldehyde for 2 days, decalcified in 14% EDTA for 3 weeks, and then incubated with 15% and 30% sucrose, serially, for 2 days. The samples were embedded for frozen sections allowing retention of both bone and resin, and the sections were cut longitudinally at 12 μm. The serial sections were stained with hematoxylin and eosin. The newly formed bone at the fracture site was evaluated.

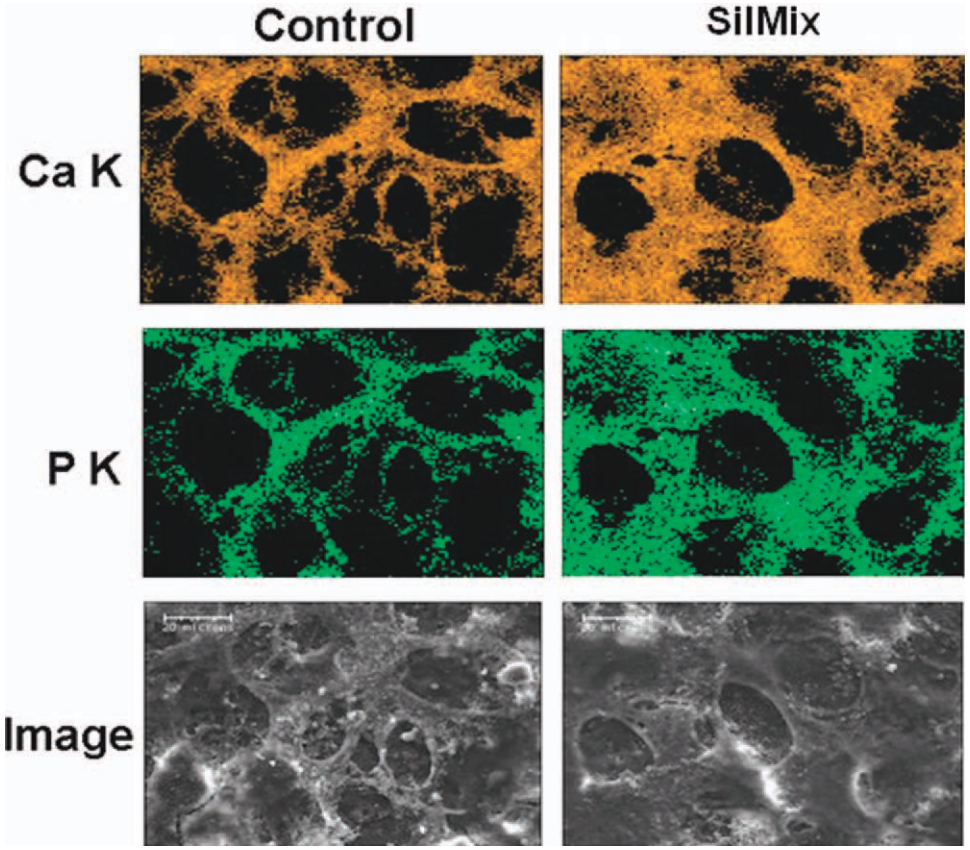

FIGURE 7. Elemental analysis (EDS) of the mineralized honeycomb-like matrix formed by the MLO-A5 cells at 11 days of culture on cover slips with resin drops. The calcium component pattern colorized as orange (Ca K) completely matches or overlays with the phosphate component pattern colorized as green (P K) and is consistent with the micrograph image (Image). Again, by this approach the cell matrix formed in the SilMix containing wells appears more mineralized (scale bars = 20 μm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Statistical Analysis

Statistical significance was determined either using the one-way ANOVA and Tukey post test or in some cases the three-way ANOVA for significance at the $p < 0.05$ level. Experiments were repeated a minimum of two times with similar results.

RESULTS

The chemical structure of the silorane that was used in this study is shown in Figure 1. The degree of polymerization of the SilMix and the BT resins can be found in Figure 2. Peak ratios of a spectral peak associated with polymerization (883 cm^{-1} representing ring-opening in siloranes) with an unchanging peak (1257 cm^{-1} in curing siloranes) were calculated for each material. For the BT specimens, degree of polymerization was calculated, based on the 1637 cm^{-1} (C=C) associated with polymerization with respect to 1714 cm^{-1} (C=O). For the first set of *in vitro* experiments, resin drops were placed either in the center or off-center in culture wells. No significant differences were observed depending on drop placement. The cell number was higher in the wells containing silorane resin drops at 24, 48, and 72 h as compared to a greater than 50% reduction in methacrylate BT containing wells [Figure 3(A)]. In contrast to the resin drop cultures, there were pronounced decreases in cell numbers for cells grown on the polymer disc surfaces [Figure 3(B)]. In order to test if this effect was due to toxic leachables, extraction of the disc resins was performed. The amount of formazan produced by cells in the presence of SilMix disc extracts was similar to levels of formazan produced by control cells (tissue culture grade polystyrene conditioned media); however, BT resin extracts generated considerable toxicity [Figure 3(C)]. This shows that no toxic component was released by the SilMix resin in contrast to the BT resin.

Using the trypan blue dye exclusion method, the number of live cells on SilMix surfaces were significantly lower ($p < 0.05$) than the controls [Figure 4(A)]. The number of dead cells were also lower but not significantly different from the number of dead cells in the controls. Upon calculation of the percent of live and dead cells, the percentage of live cells obtained with SilMix was similar to the controls [Figure 4(B)]. However, the number and percent of live cells for BT was very low at 24 h with most cells dead at 48 h.

Because the cells did not adhere well to the resin discs, the resin drop approach was used to examine osteoblast differentiation and function. Collagen type 1 is essential for

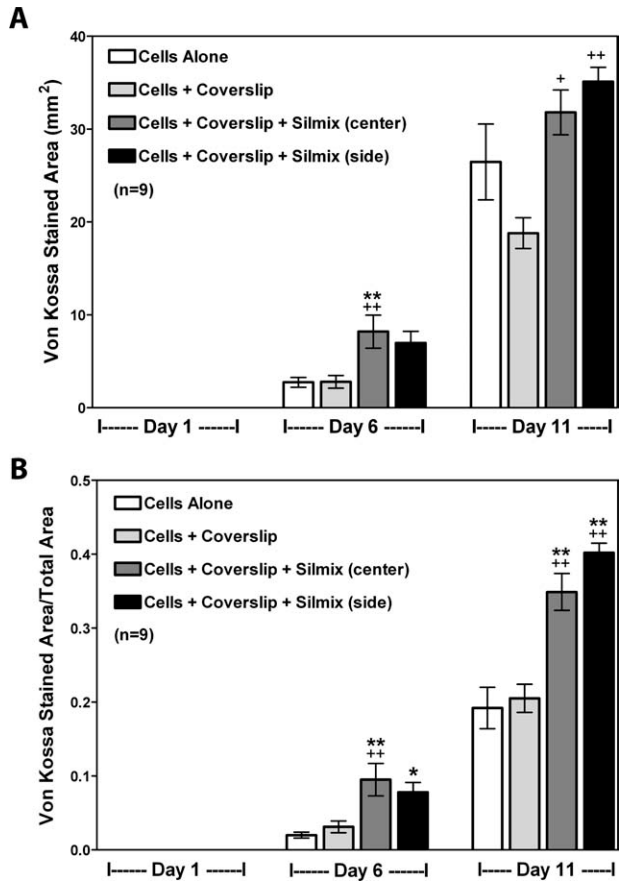

FIGURE 8. Quantitation of mineral formed in cultures on cover slips with resin drops using von Kossa staining. A good correlation is observed between each stain for phosphate in (A) and per total area measured in (B). $^{+}$Significantly different from cells + coverslip ($p < 0.05$); *significantly different from cells alone ($p < 0.05$); $^{++}$significantly different from cells + coverslip ($p < 0.01$); **significantly different from cells alone ($p < 0.01$) using one-way ANOVA and Tukey post test.

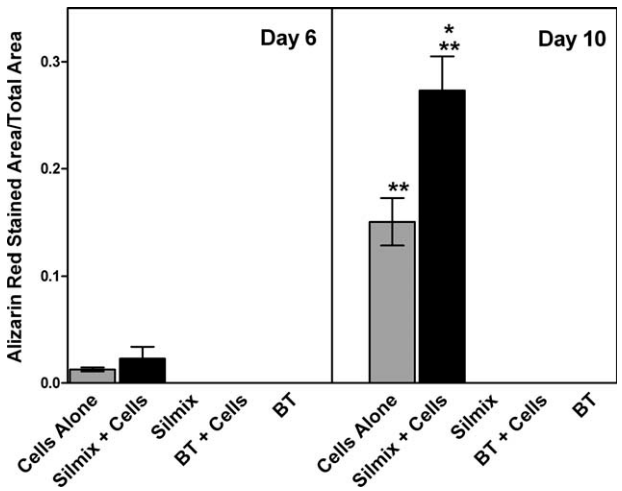

FIGURE 9. Quantitation of mineral formed in cultures using Alizarin red staining for calcium at 6 and 10 days on cover slips with resin drops. Whereas the BT treated cultures had no detectable mineral, surprisingly, the SilMix cultures had greater staining for mineral than control cultures. Neither of the cover slips with just the resins, exhibited any background staining. *Significantly different from cells alone ($p < 0.01$); **Significantly different from BT + cells ($p < 0.001$) using one-way ANOVA and Tukey post test.

the normal mineralization of bone.[28] Immunofluorescent staining for type 1 collagen was performed on MLO-A5 cells at day 6 of culture (Figure 5). The pattern is clearly fibrillar at 6 days and of greater intensity in the silorane containing culture (right panel) compared to control (left panel) [Figure 5(A)]. Alkaline phosphatase activity at 6 days was significantly higher in culture wells with silorane than in the control [Figure 5(B)].

Analysis of the ultrastructure of the cultures using SEM was also performed [Figure (6)]. The top row shows the cells after 24 h in culture. In contrast to the cells cultured

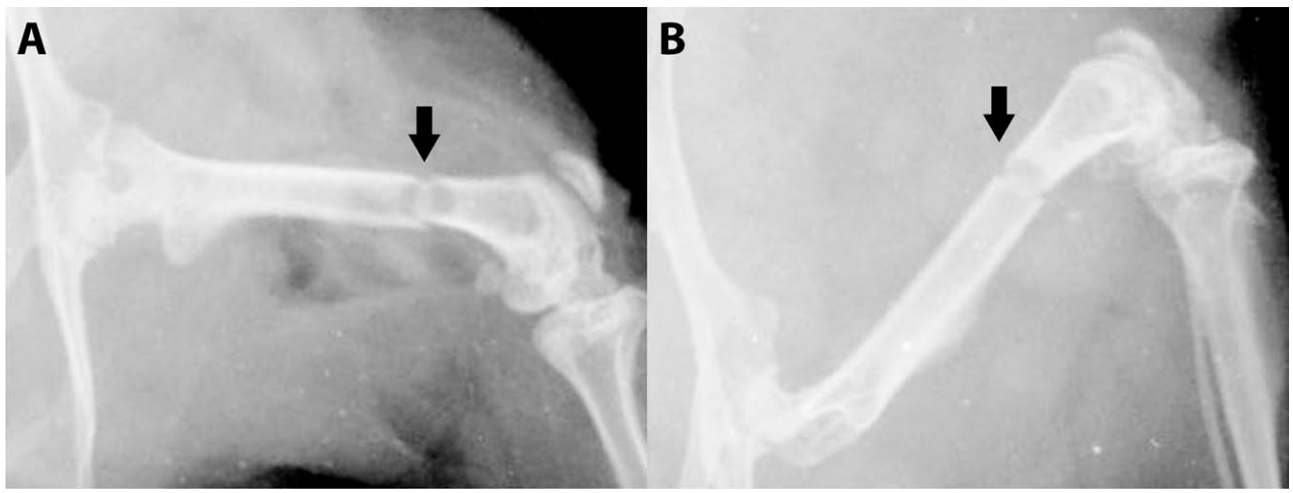

FIGURE 10. Radiographs of the SilMix resin stabilized murine femori at day 0 (A) and 28 (B) postsurgery. There was no sign of displacement of the femoral fracture (arrow). The fracture gap was coalescing at 28 days (magnification = ×2).

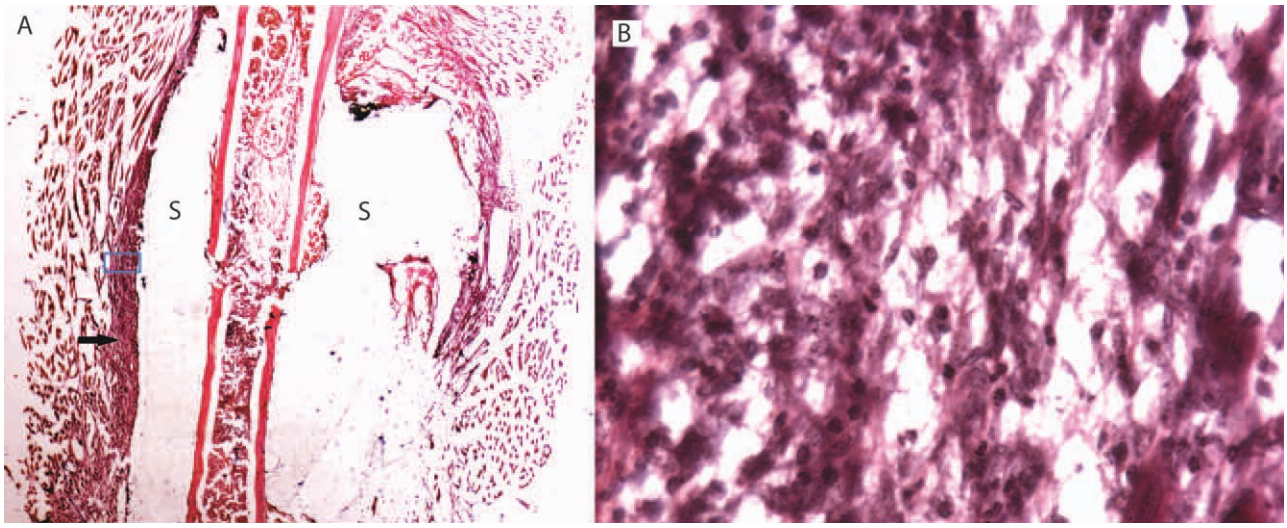

FIGURE 11. Histological section showing SilMix resin (S) encasing the murine osteotomized femur. The arrow shows granulation tissue between muscle tissue and biomaterial and filling the fracture gap (A). No inflammatory response was observed at 7 days postsurgery. The blue box is the area of magnification as reflected in B (original magnification: A, ×1; B, ×40).

in the BT-containing wells, which were dying at 24 h and absent by 48 h, the control cultures (left panel), as well as silorane containing cultures(right panel), showed high cell confluency. After 6 days under mineralization conditions, the cells in the silorane containing cultures appeared similar to controls, with a well-formed honeycomb-like matrix. These cells cultured up to 10 days showed the formation of a mineralized matrix covering the entire well. The mineralized honeycomb-like matrix formed by the MLO-A5 cells in the presence of silorane was analyzed for mineral content using energy dispersive spectroscopy (EDS) to obtain calcium and phosphorus distribution maps (Figure 7). Calcium (A) and phosphorus (B) were co-localized within the mineralized structures of the matrix (C-SEM). Quantification of mineralization was performed using both von Kossa (Figure 8) and Alizarin Red staining (Figure 9). Von Kossa detects phosphate, whereas alizarin red detects calcium.[29] As can be seen in Figure 8, von Kossa staining increased with extended time in culture in the control wells and the wells containing silorane. There was a complete absence of staining in the wells containing BT.

In these cultures in which the resin was centered in the well, the mineralized matrix appeared to build up around the silorane resin drop. Next, mineralization assays were performed on cells grown on Thermanox discs with the silorane drop placed in the center as compared to the sides of the disc. No significant effects were observed on mineralization whether quantified using the total stained area or the total stained area divided by the total area in the well which included the resin drop (Figures 8 and 9). No significant

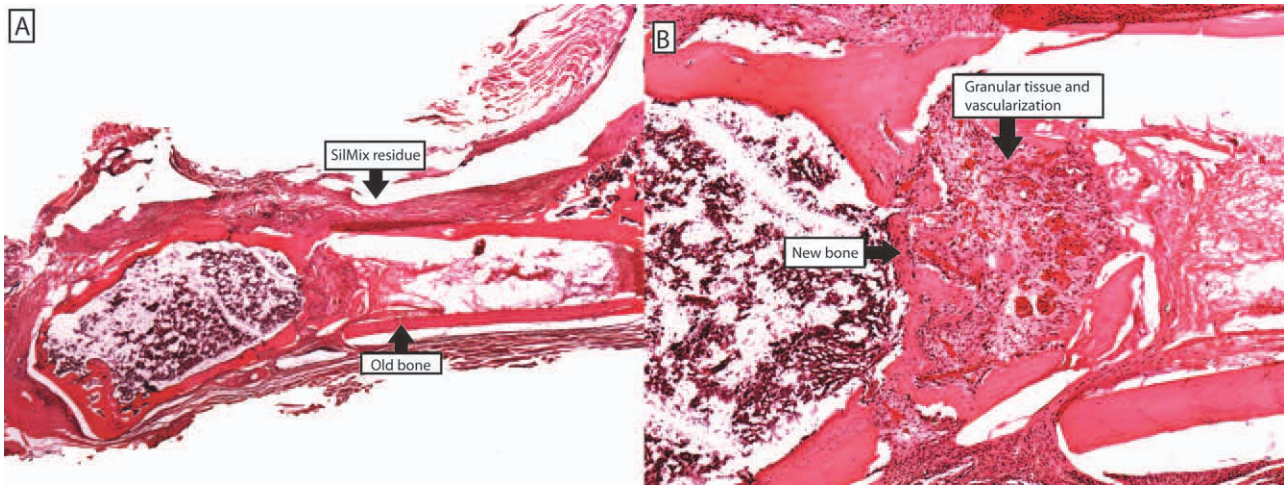

FIGURE 12. Histological section showing SilMix resin residue around the fracture site, newly formed woven bone filling the fracture gap, many blood vessels present in the newly formed woven bone area at 28 days. No inflammatory reaction was observed (original magnification: A, ×1; B, ×4).

difference was found whether the drop was placed in the center or at the side of the coverslip. Therefore, the position of the resin had no effect on cellular differentiation and mineralization.

Radiographic assessment of osteotomies of femora (transverse straight-line fracture) was performed by X-ray at days 0, 7, 14, 21, and 28 post-operation. Radiographs postsurgery showed that there was no sign of displacement of the femoral fracture at 28 days (Figure 10). The fracture gap became opaque, and no external callus was observed at 28 days postsurgery (other time points are not shown). The osteotomized femurs were encased by SilMix resin, and there was no displacement of the stabilized fractured bones. Furthermore, no inflammatory response was observed around the fracture sites (Figure 11). One to two layers of cells were observed between the bone and material at day 7 postsurgery (Figure 11). The granulation tissue containing fibroblasts and interspersed small blood vessels were seen between the muscle tissue and biomaterial. Histological evaluation showed that there was no sign of displacement of the femoral fracture at 28 days postsurgery (Figure 12). SilMix resin residue was observed around the fracture site. The fracture gap was filled by newly formed woven bone. Blood vessels were present in the newly formed woven bone area. No inflammatory reaction or external callus was observed at the fracture site.

DISCUSSION

We have previously developed silorane-based resins superior to methacrylate-based resins based on enhanced biocompatibility and significantly less polymerization stress without an associated proportional reduction in mechanical properties.[7,8] Siloranes are now being used as matrix resins to produce low stress/shrinkage dental composites with reduced cytotoxicity and genotoxicity.[12] Clearly, the siloranes are superior with respect to a lower exothermic temperature, less shrinkage, and less toxicity, compared to methacrylates representing a major step forward for use beyond dental composites. Therefore, we hypothesized that siloranes may be an improvement and serve as replacements of methacrylates used in orthopedic applications, such as bone cements, and performed *in vitro* and *in vivo* experiments to begin to test this hypothesis.

In this study, we demonstrated that silorane-based resins are nontoxic to bone cells and support parameters of bone formation both *in vitro* and *in vivo*. The mineralized, formed matrix was composed of collagen type I in a honeycomb-shaped structure, and the mineral component contained calcium and phosphate in a normal ratio compared to controls and normal bone. Surprisingly, our experiments showed that regardless of placement of silorane in culture wells the bone cells mineralized similarly, if not to a greater extent than control wells. This result may be due to the fact that the resin drop itself displaced surface area and increased cell number per area. Whereas this could explain the increase in mineralized matrix, it could not explain the increase in alkaline phosphatase. Further experiments are required to validate this observation.

Surfaces on prosthetic devices and bone cements can have either beneficial or detrimental effects on bone cells.[30] Many materials have ideal structural properties to function as implants, cements, or scaffolds but do not have the necessary biocompatibility or bioactivity. Conversely, many materials have neutral or enhancing biological properties but lack the necessary mechanical properties. Materials can be toxic, neutral, or can even support bone growth, especially with the inclusion of growth factors.[31] In this study, low bone cell attachment to silorane surfaces was observed. We have shown that surfaces can modulate the differentiation of osteoblasts,[32] and Boyan and coworkers have shown that surface roughness and microtopography plays a role in bone cell function and mineralization.[33,34] Therefore, biocompatibility and induction of bone growth become important issues. Even though low bone cell attachment was observed, this property could be improved using approaches such as surface etching.

Also, in this study, we demonstrated that when the silorane resin was placed around a bone defect, a femoral osteotomy, no inflammation was observed. Surprisingly, at 28 days, the bone began to heal in the absence of callus. This result raises the question as to whether this approach could be used in patients to stabilize bone. Pediatric orthopedic surgeons are often faced with fracture situations where the bone is either too small to support plate stabilization, or too close to the physis such that fracture stability cannot be achieved without jeopardizing the integrity of the physis. An inert stabilizer that is not toxic to physeal cartilage could be ideal in this setting. Other fracture applications might include patients with severely osteoporotic bone, where screws may not achieve good integration, or in patients with significant contractures (as in cerebral palsy or stroke) that do not allow for standard nail or plate insertion without creating further injury. Other uses include battlefield situations or natural disasters, where fractures could be stabilized before transport for more permanent treatment. This material would be easy to remove from the fracture site for definitive treatment. Therefore, in addition to being a substitute for methyl methacrylate in bone cement, silorane resin could function as a bone stabilizer. These concepts are undergoing further testing in our laboratory.

CONCLUSION

In conclusion, bisGMA/TEGDMA was toxic and totally inhibited bone cell growth while the low toxicity of silorane resin supported bone cell differentiation, matrix formation, and mineralization. In addition, all of the experimental methods, such as von Kossa and Alizarin red staining, and the SEM and EDS analyses, were in agreement and complementary with regard to quantitation and mineral composition. These studies clearly show that the silorane is superior to the bisGMA/TEGDMA with regard to support of bone cell function and has the potential to be used as a component of bone cement or as a bone-stabilizing material.

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